# 2

# Introduction to Classical Crossprotection

**Ron S. S. Fraser**

## 1. History

In the first three decades of the 20th century, it was shown that a number of plant diseases could be transmitted by infectious sap that had been passed through a bacteria-proof filter Plant virus particles had yet to be identified and characterized, and much of the research effort of plant virologists went into describing disease symptoms and studying methods of transmission. In the late 1920s, it became apparent that, when plants were deliberately inoculated with two agents causing different types of visible symptom, there could be a form of interference

Wingard *(1)* found that, in tobacco and other hosts infected with tobacco ringspot, new growth appeared that did not show any signs of disease It was not possible to cause ringspot symptoms on these leaves by a further direct inoculation Nevertheless, sap from these symptomless leaves caused ringspot when inoculated to healthy plants. McKinney *(2)* noted that tobacco plants infected with a light green mosaic (now known to be tobacco mosaic virus) did not develop further symptoms when inoculated with a yellow mosaic form In contrast, plants infected with a mild, dark green mosaic form did develop yellow symptoms when reinoculated with the yellow form.

Further work on such interactions between viruses was facilitated by the developing ability of plant virologists to discriminate between different viruses, or isolates of the same virus, using differential hosts and the emerging techniques of serology and virus particle characterization. It was recognized early that interference occurred primarily between closely related viruses, and the term "crossprotection" was applied to indicate this relatedness Indeed, crossprotection was used as one diagnostic test for relatedness between virus isolates *(3,4)*. However, more modern approaches using nucleic acid sequenc-

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

ing and advanced serological techniques have, in some cases, prompted a re-evaluation of relationships based on crossprotection studies *(5)* Further limitations to crossprotection in studying taxonomic relationships include the facts that in some pairs of related virus isolates, crossprotection operates in one order of inoculation, but not the reverse *(6,7)*, and in some other viruses, crossprotection may not operate at all *(8)*

The potential for using a protective inoculation with a mild strain of virus as a disease-control measure against chance infection by a severe strain was recognized at an early stage *(9)*, and as early as 1937 there was a report of further attenuation of a naturally occurring mild strain of potato virus Y by high-temperature treatment of infected root cultures *(10)* However, the potential for use of protective inoculations in crop protection was not rapidly taken up, and it was not until the late 1950s that mild strain protection was shown to be effective in a few crops under field conditions *(11–13)*. Today, mild strain protection occupies a small and highly specialized niche in world agriculture's defenses against plant viruses It is widely used on only a few crops, and generally other methods are preferred if available However, there are instances in which it has been of great value in crop protection. This chapter will review the practice and application of crossprotection, its merits and drawbacks, and consider possible mechanisms.

## 2. Terminology

A wide variety of terms have been used to describe phenomena of the crossprotection type. These include "interference," "acquired immunity," "antagonism," "acquired tolerance," "premunity," "cross immunization," "induced resistance," and "acquired resistance." Fulton *(14)* makes the valid point that terms based on immunity are inaccurate, because they exaggerate the level of protection generally conferred. The term crossprotection is now widely accepted for cases in which the protecting virus spreads systemically in the host. The virus involved in a second infection may be naturally occurring and transmitted, or may be deliberately introduced for experimental purposes. It may normally cause systemic or necrotic infections in the absence of crossprotection. The second virus is frequently referred to as the "challenge" inoculation or infection, and occasionally as "superinfection."

A separate pair of interactions between sequential virus inoculations are known as "localized" and "systemic acquired resistance." These occur when a plant is first inoculated with a necrotic lesion-forming virus, which does not spread systemically. The plant then appears to be resistant to a challenge inoculation by a further lesion-forming virus, because the lesions formed, either on the primarily infected leaf, or on previously uninoculated leaves, tend to be smaller or less numerous than those formed on previously untreated plants

*(15,16)*. These types of apparent resistance are highly nonspecific, they can be induced by agents as diverse as fungal and bacterial pathogens, chemicals such as salicylic acid, and plant developmental processes such as senescence and flowering *(17)* Localized and systemic acquired resistance are accompanied by accumulation of the so-called pathogenesis-related (PR) proteins, which have chitinase, β-1-3 glucanase, and other activities, and which appear to be involved in defense responses to fungal and bacterial pathogens *(18)*. However, the reality of the apparent induced resistance against a second virus infection has been questioned *(19)*, and none of the PR proteins has yet been shown to have any antiviral activity These types of resistance and the PR proteins are not considered further in this chapter.

## 3. Crossprotection Case Histories

### *3.1. Cocoa Swollen Shoot Virus*

This virus causes a very serious disease of cocoa in West Africa and is endemic in most production areas It was suggested as an early target for crossprotection *(11)*, but, despite a considerable amount of research, the method was not introduced on any scale This was largely because government policy was to attempt to eradicate the disease by removing infected plants. This policy has proved impossible to implement in practice and the disease position has, if anything, worsened. In a recent review, Hughes and Ollennu *(20)* recommend that crossprotection be re-evaluated as a strategy. They note the need for studies of the interaction between different mild strains and modern hybrid cultivars and the development of efficient methods of inoculation

### *3.2. Passion Fruit Woodiness Virus*

This disease, important in Australia, was another suggested early target for crossprotection *(12,21)*. However, despite early promise, the method does not seem to have been widely adopted *(13)*.

### *3.3. Citrus Tristeza Virus*

This virus causes serious diseases in a number of types of citrus trees (sweet and sour orange, lime, and grapefruit), and is distributed worldwide. The initial experiments on crossprotection by mild strains were carried out in Brazil *(22)* Muller and Costa *(23)* isolated a number of mild strains from trees showing no or attenuated disease symptoms and checked each for mildness and cross-protecting ability in a number of different types of citrus trees and root stocks. An important finding—a general principle for approaches to crossprotection—was that it was necessary to match each host genetic background to the most effective mild strain. A strain that crossprotected in one host would not necessarily do so in a different one. In Brazil, tens of millions of orange trees are now

protected *(24)*, and the method has been applied in India *(25)*, the Middle East *(26)*, Florida *(27)*, and South Africa *(28)*

### 3.4. Papaya Ringspot Virus

This virus is a limiting factor in the production of pawpaws in a number of areas of the world, and, at present, no resistant cultivars are available. Naturally occurring mild strains have not been readily selectable, but mutants have been produced after nitrous acid mutagenesis *(29)*. Crossprotection by these mutants was not economically beneficial under high disease pressure from plants naturally infected with the severe strain; but regular rogueing of such sources of inoculum, combined with crossprotection, provided a 111% increase in income *(30)*. Crossprotection has been introduced on a wide scale in Taiwan *(31)*, and further trials are being conducted in Mexico, Florida, Hawaii, Thailand, and Israel *(32)*. Further effort is being given to develop improved attenuated strains. A resistance gene, *Wmv* in *Cucumis metuliferus*, which is overcome by some nitrous acid-induced mild strains of papaya ringspot, but not by severe strains, has been used for further selection of attenuated cross-protecting isolates *(33)*.

### 3.5. Tomato Mosaic Virus

This virus, recognized also as a strain of tobacco mosaic virus, has caused severe disease problems in tomato. However, most modern cultivars now contain the *Tm-2*$^2$ gene for tomato mosaic resistance. This has proved highly effective and durable and resistance-breaking isolates of a virus are rare and do not readily become established. Crossprotection, especially with the nitrous acid-induced mutant MII-16 *(34)*, and by naturally occurring mild strains *(35)*, was previously quite widely used in Europe and North America, but is now mainly restricted to varieties grown for particular quality characteristics, such as flavor or size, which do not yet have the *Tm-2*$^2$ resistance gene *(36)*. Mild strain crossprotection of tomatoes caused a small depression of yield, normally around 5% *(37)*, but this compares well with potential losses of 25–50% occurring with a severe strain infection of unprotected plants.

### 3.6. Zucchini Yellow Mosaic Virus

This is probably the most recent virus to be tackled by crossprotection methods. The virus causes severe yield losses in cucurbit crops (cucumber, melon, courgette, and marrows) The fruit of infected plants are severely distorted and discolored and quite unmarketable The virus is present at a low level on the testa and inner chlorenchyma tissue of the seed coat *(38)*. This probably leads to seedling infection, which is then transmitted rapidly by aphids. In recent years, courgette growers in the UK have suffered total crop loss from this virus.

**Table 1**
**Further Examples of Effective Crossprotection Mechanisms**

| Plant host | Virus | Ref. |
|---|---|---|
| Vanilla | Vanilla necrosis potyvirus | *43* |
| Cucurbits | Water melon mosaic virus | *44* |
| Soybean | Soybean mosaic virus | *45* |
| Tomato | Tomato spotted wilt virus | *46* |
| Plum | Plum pox virus | *47* |
| Oat | Barley yellow dwarf virus | *48* |
| Pepper | Pepper severe mosaic virus | *49* |
| Peach | Tomato ringspot virus | *50* |
| Tomato | Tomato aspermy virus | *51* |
| Apple | Apple mosaic virus | *52* |
| Brussels sprout | Cauliflower mosaic virus | *53* |

A mild variant of the virus selected from a severe strain in France has been used for crop protection successfully in various cucurbits grown in a number of countries *(39–41)*. The mild strain causes a slight depression of yield and can delay flowering in early season crops *(42)*, but clearly offers considerable commercial benefits

### 3.7. Other Examples

There are many other examples of crops and viruses in which apparently effective crossprotection has been demonstrated in laboratory/greenhouse experiments or in field trials. For completeness, a number of these are listed in **Table 1**. Generally, however, these examples do not seem to have been carried forward into practical use in crop protection. Possible reasons for this are explored in the next section

## 4. Disadvantages and Advantages of Crossprotection

Generally, the comparatively low uptake of crossprotection in agricultural systems suggests that the disadvantages are seen to outweigh the advantages. There is a sensible reluctance to introduce viruses into the agricultural ecosystem, because of possible deleterious consequences, and, in general, crossprotection has only been used when other measures, such as resistance, have been unavailable, where virus eradication has failed and the target virus has become endemic, or where the release could be carried out in controlled conditions, such as in greenhouse-grown crops.

A number of potential problems with crossprotection have been considered in earlier reviews *(13,14,17)*. These are considered briefly here.

Where it has been measured, mild strains have often been shown to cause a loss of yield of 5–10% *(37,41)*, but this is considered acceptable if there is a high chance of a much greater loss caused by severe strain infection of unprotected plants.

The isolation of crossprotecting isolates may be difficult. Mild-strain (attenuated) isolates do appear to occur naturally in agriculture or wild plants *(23)*, but their selection and matching to host genotype for optimum performance can be time-consuming In other cases, it has been necessary to produce mild strains by mutagenesis with nitrous acid or UV light, or by high- or low-temperature treatment of infected plants. A deeper understanding of the molecular basis of attenuation *(54,55)* should aid in the construction of designed mild isolates

Production of adequate amounts of inoculum of the crossprotecting isolate may also be difficult, because reduction in symptom severity and effects on host-plant growth can be associated with low virus multiplication. There is also an important need for quality control of the inoculum, in order to check that the virus has not produced more severe mutants during multiplication This factor applies also to infection in the protected crop plants, although there seem to have been very few reports of these eventually developing severe infection as a result of changes in the protecting strain

Inoculation of crops with the protecting strain can be a logistical problem and would clearly pose difficulties for direct-drilled, field-grown crops Plants that are propagated in modules, then transplanted to the final growing site, such as tomatoes and cucurbits, can be conveniently inoculated at the seedling stage by spray gun and abrasive. Perennial crops, such as those propagated by budding and grafting, can be inoculated during the propagation process.

Concern has been expressed that the crossprotecting virus might interact with other unrelated viral infections of the crop to produce synergistic damaging effects There do not appear to have been reports of this, and presumably the interaction could be checked experimentally.

There is also concern that a virus introduced in one crop for crossprotection may spread to other species and possibly cause severe damage there Given the variable interaction between attenuated strains and different host genetic backgrounds *(23)*, this is a concern that needs to be taken seriously, especially for viruses such as tomato spotted wilt, which have a very wide host range The problem is perhaps less serious for those viruses, such as papaya ringspot, which have very restricted ranges *(31)*.

The various problems associated with the use of crossprotection tend to emphasize the attractiveness of the alternative approach, of developing transgenic plants expressing the CP gene to confer virus resistance. This route raises ecological, risk assessment, and regulatory issues in its own right *(56,57)*, but

clearly sidesteps some of the practical and ecological problems involved in using whole viruses for crossprotection.

Finally, the European Community Directive 91/414, aimed at securing harmonization of pesticide registration and availability in the member states, classifies microbiological biocontrol agents as pesticides For these purposes, attenuated virus strains are classified as biological pesticides and therefore require registration as such The procedures for registration are expensive and not simple. Until a degree of experience in registering biologicals as pesticides is built up, this regulatory requirement may impose a further block to the introduction of effective cross-protecting viral agents.

## 5. Possible Mechanisms of Crossprotection

Numerous theories have been advanced and there has been some spirited debate in the literature *(58,59)*. The situation is probably complicated by the fact that viruses have a number of patterns of interaction within a doubly infected plant. Experiments demonstrating one particular type of interaction do not necessarily exclude the occurrence of another For example, many systemic virus infections of plants induce the formation of dark green islands of tissue that contain few or no virus particles, but are resistant to challenge inoculation with the same virus *(60)* This mechanism of resistance is probably quite separate from crossprotection in virus-containing parts of the leaf, but has confused some of the literature on the subject.

An early theory was that the cross-protecting virus depleted certain metabolites required for virus multiplication or blocked host sites specifically involved in replication. The former explanation would seem unlikely to apply to those viruses, such as potyviruses, which only multiply to very low concentrations in the host There is a lack of specific evidence for the latter explanation

Palukaitis and Zaitlin *(61)* developed a model in which interference was at the level of the viral RNAs This involved sequestration of the (–)-strand RNA produced by the challenging virus by the excess progeny positive-sense RNA of the protecting virus.

The strongest evidence is for a central role for the CP of the protected strain in crossprotection, possibly by sequestering the nucleic acid of the challenging strain, or, more likely, by preventing its uncoating *(62,63)* Overwhelming support for the involvement of CP in crossprotection is given from the numerous examples of transgenic plants expressing the CP gene for various viruses, which show a protective effect very similar to whole virus crossprotection *(64)* (*see* Chapter 3) Earlier reports that crossprotection could be induced by protein-free virus mutants *(65)* probably involve a different mechanism of interaction *(60)* Further evidence for the operation of parallel non–CP-based mechanisms comes from the demonstration of crossprotection between viroids that lack pro-

teins ***(67)***, and from a study of interaction between cucumber mosaic viruses and their pseudorecombinants showing that the ability to crossprotect mapped on RNA-1 and RNA-2, which do not code for the viral CP ***(68)***

Finally, it should be noted that, although the evidence for a central role of CP in crop protection is very strong, the mechanism need not be confined solely to inhibition of virus uncoating. There is, for example, evidence that CP may interfere with replicase activity ***(69)***, and CP in whole virus-protected or transgenic plants may also interfere with systemic transport of the challenging virus ***(64,70)*** (*see* Chapters 52 and 53).

## References


1 Wingard, S A (1928) Hosts and symptoms of ringspot, a virus disease of plants *J Agric Res* **37,** 127–153

2 McKinney, H H (1929) Mosaic diseases in the Canary Islands, West Africa, and Gibraltar *J Agric Res* **39,** 557–578

3 Latorre, B A and Flores, V (1985) Strain identification and cross-protection of potato virus Y affecting tobacco in Chile *Plant Dis* **69,** 930–932

4 Taiwo, M A , Gonsalves, D , Provvidenti, R , and Thurston, H D (1982) Partial characterisation and grouping of isolates of Blackeye Cowpea Mosaic and Cowpea aphidborne mosaic viruses *Phytopathology* **72,** 590–596

5. Shukla, D D and Ward, C W (1989) Identification and classification of potyviruses on the basis of coat protein sequence data and serology *Arch Virol* **106,** 171–200.

6 Price, W C (1936) Specificity of acquired immunity from tobacco-ring-spot diseases. *Phytopathology* **26,** 665–675

7 Fulton, R W (1978) Superinfection by strains of tobacco streak virus *Virology* **85,** 1–8

8 Bennett, C W (1955) Recovery of water pimpernel from curly top and the reaction of recovered plants to reinoculation with different virus strains *Phytopathology* **45,** 531–536

9 Salaman, R N (1933) Protective inoculation against a plant virus *Nature* **131,** 468

10 Salaman, R N (1937) Acquired immunity against the "Y" potato virus *Nature* **139,** 924–925

11 Posnette, A F and Todd, J. McA. (1955) Virus diseases of cacao in West Africa *Annu Rev Phytopathol* **24,** 355–381

12 Simmonds, J H (1959) Mild strain protection as a means of reducing losses from the Queensland woodiness virus in the Passion vine *Queensland J Agric Sci* **16,** 371–380

13 Urban, L A , Sherwood, J L., Rezende, J A M , and Melcher, U (1990) Examination of mechanisms of cross protection with non-transgenic plants, in *Recognition and Response in Plant–Virus Interactions* (Fraser, R S S , ed ), Springer-Verlag, Berlin, pp 415–426

14. Fulton, R W (1986) Practices and precautions in the use of cross protection for plant virus disease control *Annu Rev Phytopathol* **24,** 67–81

15 Ross, A F (1961) Localized acquired resistance to plant virus infection in hypersensitive hosts *Virology* **14,** 329–339
16 Ross, A F (1966) Systemic effects of local lesion formation, in *Viruses of Plants* (Beemster, A B R and Dijkstra, J , eds ), North-Holland, Amsterdam, pp 129–150
17 Fraser, R. S S. (1985) Mechanisms of induced resistance to virus disease, in *Mechanisms of Resistance to Plant Diseases* (Fraser, R. S. S , ed ), Martinus Nijhoff/Dr W Junk, Dordrecht, pp 373–404
18 Fritig, B , Kauffmann, S., Rouster, J , Dumas, B , Geoffroy, P, Kopp, M., and Legrand, M (1990) Defence proteins, glycanhydrolases and oligosaccharide signals in plant-virus interactions, in *Recognition and Response in Plant–Virus Interactions* (Fraser, R. S S., ed.), Springer-Verlag, Berlin, pp 375–394
19. Fraser, R S S (1982) Are "pathogenesis-related" proteins involved in acquired systemic resistance of tobacco plants to tobacco mosaic virus? *J Gen Virol* **58,** 305–313
20 Hughes, J -d'A and Ollennu, L. A A. (1994) Mild strain protection of cocoa in Ghana against cocoa swollen shoot virus a review *Plant Pathol* **43,** 442–457
21 Greber, R S (1966) Passionfruit woodiness virus as the cause of passionvine tip blight disease. *Queensland J Agric Anim Sci* **23,** 533–536
22 Grant, T J and Costa, A S (1951) A mild strain of the tristeza virus of citrus *Phytopathology* **41,** 114–122
23 Muller, G W and Costa, A S (1977) Tristeza control in Brazil by preimmunization with mild strains *Proc Int Soc Citricult* **3,** 868–872
24 Salibe, A. A. (1987) Clones nucelares de citros no Estado de São Paulo *Laranja* **2,** 443–466
25 Balaraman, K (1981) Preliminary progress report of acid lime seedlings inoculated with mild strain of citrus tristeza virus *Phytopathol Mediterr* **20,** 149–151
26 Bar-Joseph, M , Roistacher, C N , Garnsey, S M , and Gumpf, D J (1982) A review on tristeza, an ongoing threat to citriculture. *Proc Int Soc Citricult* **1,** 419–423
27. Adams, S (1988) Orlando lab puts fresh squeeze on Florida citrus problems *Agric Res USA* **36,** 6–10
28. Van Vuuren, S P, Collins, R P, and da Graca, J V (1993) Evaluation of citrus tristeza virus isolates for cross protection of grapefruit in South Africa *Plant Dis* **77,** 24–28
29. Yeh, S. D , Wang, H L., and Chiu, R J. (1986) Control of papaya ringspot virus by seedling inoculation with mild virus strains, in *Plant Virus Diseases of Horticultural Crops in the Tropics and Subtropics* Food and Fertilizer Technology Centre, Taiwan, pp 169–178
30 Wang, H L., Yeh, S D , Chiu, R J , and Gonsalves, D (1987) Effectiveness of cross-protection by mild mutants of papaya ringspot virus for control of ringspot disease of papaya in Taiwan *Plant Dis* **71,** 491–497
31. Yeh, S. D (1990) Control of papaya ringspot virus by cross protection conventional versus innovative Integrated control of plant virus diseases, in *Proceedings*

*of the International Workshop TARI* (Kiritani, K , Su, H J , and Chu, Y I , eds ), Taichung, Taiwan pp 125–134

32 Yeh, S D., Gonsalves, D , Wang, H L , Namba, R , and Chiu, R J (1988) Control of papaya ringspot virus by cross protection *Plant Dis* **72,** 375–380

33 Yeh, S D and Cheng, Y. H (1989) Use of resistant Cucumis metuliferus for selection of nitrous-acid induced attenuated strains of papaya ringspot virus *Phytopathology* **79,** 1257–1261

34 Rast, A. T B (1975) Variability of tobacco mosaic virus in relation to control of tomato mosaic in glasshouse crops by resistance breeding and cross protection *Agric Res. Rep Wageningen* (Netherlands) **834,** 1–76

35. Ahoonmanesh, A. and Shalla, T A (1981) Feasibility of crossprotection for control of tomato mosaic virus in fresh market field-grown tomatoes *Plant Dis* **65,** 56,57

36 Fletcher, J. T (1992) Disease resistance in protected crops and mushrooms *Euphytica* **63,** 33–49

37 Channon, A G , Cheffins, N J , Hitchon, G. M , and Barker, J (1978) The effect of inoculation with an attenuated mutant strain of tobacco mosaic virus on the growth and yield of early glasshouse tomato crops *Ann Appl Biol* **88,** 121–129

38 Walkey, D G A (1992) Zucchini yellow mosaic virus control by mild strain protection *Phytoparasitica* **20,** 99–103

39 Wang, H L., Gonsalves, D , Provvidenti, R , and Lecoq, H L (1991) Effectiveness of cross protection by a mild strain of zucchini yellow mosaic virus in cucumber, melon and squash *Plant Dis* **75,** 203–207

40 Lecoq, H , Lemaire, J M , and Wipf-Scheibel, C (1991) Control of zucchini yellow mosaic virus in squash by cross protection *Plant Dis* **75,** 208–211

41 Walkey, D G A (1992) Studies on the control of zucchini yellow mosaic virus in courgettes by mild strain protection *Plant Pathol* **41,** 762–771

42 Spence, N J and Walkey, D G A (1993) Mild strain cross protection to control zucchini yellow mosaic virus *Horticulture Res Int Ann Rep 1992/93*, pp 68,69

43 Liefting, L W , Pearson, M. N , and Pone, S P (1992) The isolation and evaluation of two naturally occurring mild strains of vanilla necrosis potyvirus for control by cross protection *J Phytopathol* **136,** 9–15

44 Kameya-Iwaki, M , Tochihara, H , Handa, K , and Torigoe, H Attenuated isolate of watermelon mosaic virus (WMV-2) and its cross protection against virulent isolate *Ann Phytopathol Soc Japan* **58,** 491–494

45 Kosaka, Y and Fukunishi, T (1993) Attenuated isolates of soybean mosaic virus derived at low temperature *Plant Dis* **77,** 882–886

46 Wang, M and Gonsalves, D (1992) Artificial induction and evaluation of a mild isolate of tomato spotted wilt virus. *J Phytopathol* **135,** 233–244

47 Rankovic, M. and Paunovic, S (1989) Further studies on the resistance of plums to sharka (plum pox) virus *Acta Hort* **235,** 283–290

48. Wen, F , Lister, R M , and Fattouh, F A (1991) Cross protection among strains of barley yellow dwarf virus. *J Gen Virol* **72,** 791–799

49. Tanzi, M , Betti, L , and Canova, A (1988) Protection of pepper crops under glass against PepMV artificial infection with the mild mutant M-1 analysis of its influence on growth and yield *Adv Hort Sci* **1,** 19–22.
50 Bitterlin, M W , Gonsalves, D , and Scorza, R (1987) Improved mechanical transmission of tomato ringspot virus to Prunus seedlings *Phytopathology* **77,** 560–563
51. Kuti, J O and Moline, H E (1986) Effects of inoculation with a mild strain of tomato aspermy virus on the growth and yield of tomatoes and the potential for cross protection. *J Phytopathol.* **115,** 56–60
52 Chamberlain, E. E , Atkinson, J. D., and Hunter, J A (1964) Cross protection between strains of apple mosaic virus *N Z J Agric Res* **7,** 480–490
53 Tomlinson, J A and Shepherd, R J. (1978) Studies on mutagenesis and cross-protection of cauliflower mosaic virus *Ann Appl Biol* **90,** 223–231
54 Stratford, R , Plaskitt, K A , Turner, D. S , Markham, P. G , and Covey, S N (1988) Molecular properties of Bari 1, a mild strain of cauliflower mosaic virus *J Gen Virol* **9,** 2375–2386
55 Holt, C A , Hodgson, R A J , Coker, F A , Beachy, R N , and Nelson, R S (1990) Characterisation of the masked strain of tobacco mosaic virus identification of the region responsible for symptom attenuation by analysis of an infectious cDNA clone *Mol Plant Microbe Interact* **3,** 417–423
56 Hull, R (1990) The use and misuse of viruses in cloning and expression in plants, in *Recognition and Response in Plant-Virus Interactions* (Fraser, R S S , ed ), Springer-Verlag, Berlin, pp 443–457
57 DeZoeten, G A (1991) Risk assessment do we let history repeat itself? *Phytopathology* **81,** 585–586
58 DeZoeten, G A and Fulton, R W (1975) Understanding generates possibilities *Phytopathology* **65,** 221,222
59 Zaitlin, M (1976) Viral cross-protection more understanding is needed *Phytopathology* **66,** 382–383
60 Whenham, R J and Fraser, R S S (1990) Plant growth regulators, viruses and plant growth, in *Recognition and Response in Plant–Virus Interactions* (Fraser, R S S , ed ), Springer-Verlag, Berlin, pp 287–310
61 Palukaitis, P and Zaitlin, M (1984) A model to explain the cross protection phenomenon shown by plant viruses and viroids, in *Plant-Microbe Interactions Molecular and Genetic Perspectives* (Kosuge, T and Nester, E W , eds.), MacMillan, New York, p 420
62 Wilson, T M A and Watkins, A C. (1986) Influence of exogenous viral coat protein in the cotranslational disassembly of tobacco mosaic virus (TMV) particles in vitro *Virology* **149,** 132–135
63 Osbourn, J. K , Plaskitt, K A , Watts, J W., and Wilson, T M A (1989) Tobacco mosaic virus coat protein and reporter gene transcripts containing tobacco plants, implications for coat protein-mediated protection *Molec Plant Microbe Interact* **2,** 340–345.
64 Nelson, R S , Powell, P A , and Beachy, R N. (1990) Coat protein-mediated protection against virus infection, in *Recognition and Response in Plant–Virus Interactions* (Fraser, R S S , ed ), Springer-Verlag, Berlin, pp 427–442

65 Gerber, M and Sarkar, S. (1989) The coat protein of tobacco mosaic virus does not play a significant role for cross protection *J Phytopathol* **124,** 323–331
66 Sherwood, J L (1987) Demonstration of the specific involvement of coat protein in tobacco mosaic virus (TMV) cross protection using a TMV coat protein mutant *J Phytopathol* **118,** 358–362.
67. Niblett, C. L., Dickson, E , Fernow, K. H., Horst, R K., and Zaitlin, M. (1978) Cross protection among four viroids *Virology* **91,** 198–203
68 Aranyi-Rehak, M and Burgyan, J (1992) Cross protection between cucumoviruses and their pseudorecombinants. II Changes in nucleic acids *Novenytermeles* **41,** 211–221
69 Horikoshi, M , Nakayama, M , Yamaoka, N , Furusawa, I , and Shishiyama, J (1987) Brome mosaic virus coat protein inhibits viral RNA synthesis in vitro *Virology* **158,** 15–19
70 Rezende, J. A M , Urban, L , Sherwood, J L., and Melcher, U (1992) Host effect on cross protection between two strains of tobacco mosaic virus *J Phytopathol* **136,** 147–153

# 3

# History of Coat Protein-Mediated Protection

**Eric D. Miller and Cynthia Hemenway**

## 1. Introduction

Since the first demonstration by Powell-Abel et al. *(1)* that plants engineered to express the tobacco mosaic virus (TMV) coat protein (CP) gene can resist corresponding viral infection, a decade of research on CP-mediated protection (CPMP) has produced transgenic plants resistant to a multitude of different plant viruses. This field rapidly progressed from testing resistance in model plant systems under growth chamber conditions to conducting field trials on agronomically significant crops such as tomato, potato, sugarbeets, melons, cucumber, tobacco, and rice In addition, this approach to protection has been extended by expression of other viral sequences corresponding to satellite RNAs, antisense transcripts, sense transcripts, defective interfering sequences, nonstructural genes, portions of genes, and mutated genes in transgenic plants.

The general process for development of protected plants is similar in all cases: cloning of the appropriate viral gene, transformation of selected host and identification of primary transformants, and testing for protection against infection Although the features and extent of protection conferred in each case differ, the overwhelming conclusion is that accumulation of CP and/or CP transcripts expressed in transgenic plants inhibits the normal course of challenge virus infection

## 2. Overview of Early Experiments

Early experiments on TMV *(1,2)*, alfalfa mosaic virus (AlMV) *(3,4)*, potato virus X (PVX) *(5)*, and cucumber mosaic virus (CMV) *(6)* demonstrated that plants expressing easily detectable levels of the respective CP were protected against infection. Generally, transgenic plants were assayed for CP by Western analysis and expressors were utilized in protection tests. Protection tests usu-

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

ally involved inoculation of 10–20 plants from self-fertilized progeny with different concentrations of viral inoculum. Plants were monitored for symptom development and analyzed for presence of viral CP production by Western, ELISA, or probing of dot-blots (*see* Chapters 46 and 47). Sometimes, they were also analyzed for presence of infectious virus by inoculation of extracts derived from protected transgenic plants onto new plants (*see* Chapter 49). Typically, the protection phenotype was delay in symptom development, reduction in symptoms on inoculated leaves, decrease or absence of systemic movement, and reduced virus accumulation The extent of protection was related to the levels of CP expressed in transgenic plants and the inoculum concentration used in protection experiments.

van Dun et al. *(7)* and Powell et al *(8)* showed that transgenic plants expressing translationally defective transcripts of AlMV or TMV CP genes, respectively, were not protected from infection, indicating that protection was caused by the protein rather than the transcript. Thus, the general concensus among researchers at the time was that CP levels were associated with the extent of protection. However, initial experiments on potyviral CP systems indicated that plants with very low or nondetectable levels of CP were protected, as were plants expressing only transcripts *(9–12)*. This issue of correlations between transgene expression and protection, which is discussed below, indicates that there are multiple mechanisms involved in the protection phenotype that may reflect entry, replication, and movement mechanisms for each virus.

## 3. Range of Protection

Protection has been demonstrated in 10 different plant hosts transformed with CP or nucleocapsid protein (NCP) genes derived from 14 groups of plant viruses. As indicated in **Table 1**, most examples of protection are conferred against closely related viruses. Generally, the highest level of protection is against the same virus or closely related strains from which the transgene was derived. Barker et al. *(13)* determined that combining potato leaf-roll virus (PLRV) CP and host resistance genes in potato gave additive effects on protection against PLRV infection Stark et al *(9)* first described a broader resistance in plants expressing soybean mosaic virus (SMV) CP that were protected against another potyvirus, tobacco etch virus (TEV). Ling et al. *(14)*, Namba et al. *(15)*, and Murry et al. *(16)* also reported that transgenic plants expressing potyviral CP genes were protected against heterologous potyviruses. For tobamoviruses, Nejidat and Beachy *(17)* reported that protection was effective against different viruses in this group when the CP of the challenge virus exhibited at least 60% homology to the TMV-U1 CP expressed in transgenic tobacco.

**Table 1**
**Examples of Coat Protein-Mediated Protection**

| Virus group | Virus[a] | Chimeric gene[b] | Transgenic plant | Challenge virus[c] | Ref |
|---|---|---|---|---|---|
| Alfamo | AlMV | 35S-CP-nos | Tobacco | AlMV | *4* |
| | | | Tomato | AlMV | *64* |
| | | 19S-CP-35S | Tobacco | AlMV | *3* |
| | | 35S-CP-T-DNA | Alfalfa | AlMV | *65* |
| | | | Tobacco | AlMV | *66* |
| Carla | PVS | 35S-CP-nos | Potato | PVS, PVM | *67* |
| Cucumo | CMV | 35S-CP-rbcS | Tobacco | CMV | *6* |
| | | | Cucumber | CMV | *24* |
| | CMV | 35S-CP-35S | Tobacco | CMV | *68, 69* |
| | CMV | 35S-CP-nos | Tobacco | CMV | *39* |
| | | | | CMV, CMMV | *70* |
| Furo | BNYVV | 35S-CP-nos | Sugarbeet | BNYVV | *71* |
| Ilar | TSV | 35S-CP-nos | Tobacco | TSV | *4* |
| Luteo | PLRV | 35S-CP-nos | Potato | PLRV | *25, 26, 38, 72* |
| Nepo | ArMV | 35S-CP-nos | Tobacco | ArMV | *73* |
| | GCMV | 35S-CP-nos | Tobacco | GCMV | *74* |
| Potex | PAMV | 35S-CP-nos | Tobacco | PAMV | *75* |
| | PVX | 35S-CP-rbcS | Tobacco | PVX | *5* |
| | | | Potato | PVX | *28, 21* |
| | | 35S-CP-nos | Potato | PVX | *76* |
| | | Ext-CP-nos | Potato | PVX | *77* |
| Poty | MDMV | 35S-CP-nos | Sweet corn | MDMV, MCMV | *16* |
| | PPV | 35S-CP-35S | Tobacco | PPV | *78* |
| | PRV | 35S-CMV/PRV-35S | Tobacco | TEV, PeMV, PVY, PRV | *14* |
| | | 35S-CP-nos | Papaya | PRV | *79* |
| | PVY | 35S-CP-rbcS | Potato | PVY | *28, 21* |
| | | | Tobacco | PVY | *32* |
| | | 35S-CP-nos | Tobacco | PVY | *30* |
| | | 35S-CP-nos | Tobacco | PVY | *31* |
| | SMV | 35S-CP-nos | Tobacco | SMV, TEV, PVY | *9* |
| | TEV | 35S-CP-nos | Tobacco | TEV | *10* |
| | WMV II | 35S-CP-35S | Tobacco | WMV II, PVY, TEV, BYMV, PeaMV, CYVV, PeMV | *15* |
| | ZYMV | 35S-CP-35S | Muskmelon | ZYMV | *80* |
| | | | Tobacco | WMV II, PVY, TEV, BYMV, PeaMV, CYVV, PeMV | *15* |

*(continued)*

**Table 1 *(continued)***

| Virus group | Virus[a] | Chimeric gene[b] | Transgenic plant | Challenge virus[c] | Ref |
|---|---|---|---|---|---|
| Tenui | RSV | 35S-CP-nos | Rice | RSV | *27* |
| Tobamo | TMV | 35S-CP-nos | Tobacco | TMV | *1, 2* |
| | | | Tomato | ToMV, TMV | *81* |
| | | | Tobacco | ORSV, PMMV, TMGMV | *17* |
| | | PAL-CP-nos | Tobacco | TMV | *82* |
| | ToMV | 35S-CP-rbcS | Tomato | ToMV | *81* |
| Tobra | TRV | 35S-CP-nos | Tobacco | TRV, PEBV | *83* |
| | | | Tobacco | TRV | *84* |
| Tombus | CyRSV | 35S-CP-nos | Tobacco | CyRSV | *52* |
| Tospo | TSWV | 35S-NCP-nos | Tobacco | TSWV | *19, 85, 86* |
| | | | Tobacco | TSWV, INSV | *34* |
| | | | | TSWV, INSV, GRSV | *20* |
| | | Triple gene[d] | Tobacco | TSWV, INSV, GRSV | *22* |

[a]Abbreviations AlMV, alfalfa mosaic virus, ArMV, arabis mosaic virus, BNYVV, beet necrotic yellow vein virus, BYMV, bean yellow mosaic virus, CMMV, chrysanthemum mild mottle virus, CMV, cucumber mosaic virus, CYVV, clover yellow vein virus, CyRSV, cymbidium ringspot virus, GCMV, grapevine chrome mosaic virus, GRSV, groundnut ringspot virus, INSV, impatiens necrotic spot virus, MCMV, maize chlorotic mottle virus, MDMV, maize dwarf mosaic virus, ORSV, odontoglossum ringspot virus, PAMV, potato aucuba mosaic virus, PeaMV, pea mosaic virus, PEBV, pea early browning virus, PeMV, pepper mottle virus, PLRV, potato leaf-roll virus, PMMV, pepper mild mottle virus, PPV, plum pox virus, PRV, papaya ringspot virus, PVM, potato virus M, PVS, potato virus S, PVX, potato virus X, PVY, potato virus Y, RSV, rice stripe virus, SMV, soybean mosaic virus, TEV, tobacco etch virus, TMGMV, tobacco mild green mosaic virus, TMV, tobacco mosaic virus, ToMV, tomato mosaic virus, TRV, tobacco rattle virus, TSV, tobacco streak virus, TSWV, tomato spotted wilt virus, WMVII, watermelon mosaic virus II, and ZYMV, zucchini yellow mosaic virus

[b]Corresponds to promoter-gene-3' end 35S-, 35S promoter from cauliflower mosaic virus (CaMV), nos, nopaline synthase 3' end, 19S, 19S promoter from CaMV, -35S, 35S 3' end from CaMV, T-DNA, 3' end from Agrobacterium T-DNA 25S gene, rbcS, ribulose 1,5 *bis*-phosphate carboxylase small subunit 3' end, Ext, extensin gene promoter, PAL, Phenylalanine ammonia lyase gene 2 from *Phaseolus vulgaris*

[c]Viruses for which protection was observed

[d]Triple gene 35S-TSWV NCP-nos, 35S-INSV NCP-nos, 35S-GRSV NCP-nos in one transformation vector

Resistance to tospovirus infection by expression of NCP genes in transgenic plants has been very successful. It appears that resistance to the homologous virus in plants expressing the tomato spotted wilt virus (TSWV) NCP gene may be related to the levels of NCP transcript ***(18)*** and not directly to protein

levels; resistance to heterologous tospoviruses appears to correspond with protein levels ***(19,20)***.

An alternative approach to achieving broader resistance is transformation of multiple CP genes within one plant expression vector. Lawson et al. ***(21)*** observed protection against PVX and potato virus Y (PVY) in transgenic potatoes expressing both CP genes More recently, Prins et al. ***(22)*** transformed NCPs from TSWV, impatiens necrotic spot virus (INSV), and groundnut ringspot virus (GRSV), each under separate regulation in cauliflower mosaic virus (CaMV) 35S promoter/nopaline synthase (nos) 3' end cassettes, into tobacco. They obtained a transgenic line expressing the three genes that exhibited high levels of resistance to all three viruses.

Many of the systems analyzed for CP protection relied on mechanical inoculation of test plants. Other experiments involved inoculation by vectors, which more closely approximated the natural course for infection CPMP was observed against aphid transmission of CMV ***(23,24)***, PLRV ***(25,26)***, and PVY ***(21)*** Resistance to the planthopper-transmitted rice stripe virus (RSV) was also demonstrated using CP technology ***(27)*** In contrast, expression of tobacco rattle virus (TRV) CP gene in transgenic tobacco conferred protection against mechanical inoculation with TRV, but protection was not observed against viruliferous vector nematodes

## 4. Relationship Between Transgene Expression Levels and Degree of Protection

Although some of the earlier experiments with TMV, PVX, CMV, and AlMV exhibited a correlation between extent of protection and CP levels (*see* Part VI of this volume), this has not been observed in many cases Several reports on potyviral systems indicated that CP levels were generally very low in plants expressing potyviral CP sequences, and frequently the lowest expressors were the best protected ***(9,21,28–31)*** Similar results have been reported in other systems (*see* Part VI of this volume). In addition, protection was observed in plants transformed with PLRV CP, although the protein could not be detected in transgenic plants ***(25,26)***. It remains to be determined if the lower expression levels of proteins in some systems reflects a technical difficulty with expression of certain genes, stability of the protein products, or absence of viral factors required for expression and/or stability. Although most reports involve genes expressed under the control of the CaMV 35S promoter, parameters relating to expression of genes, such as copy number and position effects may contribute to differences observed in the various systems

Additional reports on potyviruses indicated that protection was better in plants expressing untranslatable TEV CP transcripts than in plants expressing CP ***(10–12,32,33)*** Dougherty et al. ***(33)*** proposed that protection is because of

**Table 2**
**Examples of Protection Mediated by Other Viral Sequences**

| Type of sequence/gene | Virus[a] | Ref. |
|---|---|---|
| Antisense | | |
| Coat protein | CMV | *6,39* |
| | PLRV | *38* |
| | PVX | *5* |
| | PVY | *12* |
| | TEV | *10* |
| | TSWV | *18,34* |
| Intercistronic region | BMV | *40* |
| 5' or 3' Ends of RNAs | CMV | *35* |
| 3' End of genome | TMV | *36* |
| 5' End of genome | TMV | *41* |
| Coding regions | TGMV | *37* |
| Satellite | CMV | *39,42,43* |
| | TRV | *44* |
| Defective-interfering (DI) sequences | ACMV | *46* |
| | BMV[b] | *47* |
| Sense transcripts | | |
| Untranslatable CP gene | PVY | *12,32* |
| | TEV | *10,11* |
| | TSWV | *34* |
| 3' end of genome | TYMV | *45* |
| Nonstructural genes | | |
| Replicase | AlMV | *54* |
| | CMV | *49* |
| | CyRSV | *52* |
| | PEBV | *51* |
| | PVX | *50,87* |
| | PVY | *53* |
| | TMV | *48* |
| Movement Proteins | TMV | *58* |
| | WClMV | *57* |
| Protease | PVY | *56* |
| | TVMV | *55* |

[a]Abbreviations ACMV, African cassava mosaic virus, BMV, brome mosaic virus, TGMV, tomato golden mosaic virus, TVMV, tobacco vein mottling virus, TYMV, turnip yellow mosaic virus, and WClMV, white clover mosaic virus
[b]Protection observed in protoplasts

a cellular pathway that targets aberrant RNAs for elimination. Similar conclusions were made by Pang et al. *(34)* when transgenic tobacco expressing untranslatable or antisense TSWV NCP were protected against TSWV infection. Consequently, correlation, or lack thereof, between CP or transcript levels

and extent of protection may reflect differences in mechanisms of protection in response to different virus systems (further discussed in Chapters 52 and 53).

## 5. Protection Conferred by Expression of Other Viral Genes and Sequences

A few years after development of CPMP, transgenic plants were also engineered to express other viral sequences and genes (**Table 2**), including antisense RNAs *(5,6,10,11,18,32,34–41)*, satellite RNAs *(39,42-44)*, sense transcripts *(12,32,34,45)*, defective-interfering (DI) sequences *(46,47)*, replicase genes *(48–54)*, protease genes *(55,56)*, and movement protein genes *(57,58)*. Several reviews have been published on these strategies *(59–63)*.

As with CPMP, transgenic plants expressing other viral sequences display resistance phenotypes ranging from delay in symptom development to apparent immunity. In addition, discussions on mechanisms of protection also focus on potential correlations between transgene expression levels and extent of protection The diversity of protection phenotypes in response to different genes and sequences offers multiple choices for engineering virus resistance into desired crops, but compounds the problem of interpretating mechanisms associated with genetically engineered resistance

## 6. Summary

A decade of research has proven that plants can be genetically engineered to resist virus infection through expression of viral CP genes, as well as other viral genes and sequences. Additional opportunities for development of resistant plants will require research focused on mechanisms of protection, improvements in expression vector design, and transformation of new crop species. As each of these technologies is utilized singly or in combination to generate resistant crop varieties, the full impact of such engineered resistance will be realized.

## References

1. Powell-Abel, P A., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R T., and Beachy, R. N (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene *Science* **232,** 738–743
2. Nelson, R S , McCormick, S M , Delannay, X , Dube, P., Layton, J., Anderson, E. J , Kaniewski, M , Proksch, R K., Horsch, R. B , Rogers, S G , Fraley, R T , and Beachy, R N (1988) Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus *Biotechnology* **6,** 403–409
3. Loesch-Fries, L S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K , Jarvis, N., Nelson, S., and Halk, E. (1987) Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. *EMBO J* **6,** 1845–1851

4 van Dun, C M P, Bol, J F, and van Vloten-Doting, L (1987) Expression of alfalfa mosaic virus and tobacco streak virus protein genes in transgenic tobacco plants *Virology* **159,** 299–305
5 Hemenway, C, Fang, R -X, Kaniewski, W, Chua, N -H, and Turner, N E (1988) Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA *EMBO J* **7,** 1273–1280.
6 Cuozzo, M, O'Connell, K M, Kaniewski, W, Fang, R -X, Chua, N -H, and Turner, N E (1988) Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA *Biotechnology* **6,** 549–547
7 van Dun, C M P, Overduin, B, van Vloten-Doting, L, and Bol, J F (1988) Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosic virus infection *Virology* **164,** 383–389
8. Powell, P A, Sanders, P R, Turner, N E., Fraley, R T, and Beachy, R N (1990) Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences *Virology* **175,** 124–130
9 Stark, D. M. and Beachy, R. N (1989) Protection against potyvirus infection in transgenic plants evidence for broad spectrum resistance *Biotechnology* **7,** 1257–1262
10 Lindbo, J A. and Dougherty, W G (1992) Pathogen derived resistance to a potyvirus immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence *Mol Plant–Microbe Interact* **4,** 247–253
11 Lindbo, J A and Dougherty, W G. (1992) Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plant and protoplasts *Virology* **189,** 725–733
12 Smith, H A, Swaney, S L, Parks, T D, Wernsman, E A, and Dougherty, W G (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs expression, regulation, and fate of nonessential RNAs *Plant Cell* **6,** 1441–1453
13. Barker, H, Webster, K. D., Jolly, C. A., Reavy, B, Kumar, A., and Mayo, M A (1994) Enhancement of resistance to potato leafroll virus multiplication in potato by combining the effects of host genes and transgenes *Mol Plant–Microbe Interact* **7,** 528–530
14. Ling, K., Namba, S., Gonsalves, C, Slightom, J L, and Gonsalves, D (1991) Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene *Biotechnology* **9,** 752–758
15 Namba, S, Ling, K, Gonsalves, C, Slightom, J L, and Gonsalves, D (1992) Protection of transgenic plants expressing the coat protein gene of watermelon mosaic virus II or zucchini yellow mosaic virus against six potyviruses *Phytopathology* **82,** 940–946.
16. Murry, L. E, Elliot, L G, Capitant, S A, West, J A, Hanson, K K, Scarafia, L, Johnston, S, DeLuca-Flaherty, C., Nichols, S., Cunanan, D, Dietrich, P. S, Mettler,

S. D., Warnick, D A., Rhodes, C , Sinibaldi, R M., and Brunke, K. J. (1993) Transgenic corn plants expressing MDMV strain B coat protein are resistant to mixed infections of maize dwarf mosaic virus and maize chlorotic mottle virus. *Biotechnology* **11,** 1559–1564

17 Nejidat, A. and Beachy, R N (1990) Transgenic tobacco plants expressing a tobacco virus coat protein gene are resistant to some tobamoviruses *Mol Plant-Microbe Interact* **3,** 247–251

18 de Haan, P , Gielen, J J L , Prins, M , Wijkamp, M. G , van Schepen, A., Peter, D , van Ginsven, M. Q J M , and Goldbach, R. W (1992) Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants *Biotechnology* **10,** 1133–1137

19 Pang, S -Z , Nagpala, P , Wang, M , Slightom, J L , and Gonsalves, D (1992) Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene *Phytopathology* **82,** 1223–1229

20 Vaira, A M., Semeria, L , Crespi, S , Lisa, V , Allavena, A., and Accotto, G P (1995) Resistance to tospoviruses in Nicotiana benthamiana transformed with the N gene of tomato spotted wilt virus. correlation between transgene expression and protection in primary transformants *Mol Plant–Microbe Interact* **8,** 66–73

21. Lawson, C., Kaniewski, W., Haley, L., Rozman, R , Newell, C., Sanders, P., and Tumer, N E (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank *Biotechnology* **8,** 127–134.

22 Prins, M., de Haan, P., Luyten, R , van Veller, M., van Ginsven, M Q. J M , and Goldbach, R (1995) Broad resistance to tospoviruses in transgenic tobacco plants expressing three tospoviral nucleoprotein gene sequences *Mol Plant–Microbe Interact* **8,** 85–91

23 Quemada, H D , Gonsalves, D , and Slightom, J. L (1991) Expression of coat protein gene from cucumber mosaic virus strain C in tobacco· protection against infections by CMV strains transmitted mechanically of by aphids *Phytopathology* **81,** 794–801

24 Slightom, J. L , Chee, P P , and Gonsalves, D (1990) Field testing of cucumber plants which express the CMV coat protein gene· field plot design to test natural infection pressures, in *Progress in Plant Cell and Molecular Biology* (Nijkamp, H J J , van der Plass, L H W , and van Aartrijk, J., eds ), Kluwer, The Netherlands, pp 201–206

25 Kawchuk, L M., Martin, R R , and McPherson, J (1990) Resistance in transgenic potato expressing the potato leafroll virus coat protein gene *Mol Plant-Microbe Interact* **3,** 301–307

26. van der Wilk, F., Willink, D. P-L., Huisman, M J , Huttinga, H , and Goldbach, R (1991) Expression of the potato leafroll luteovirus coat protein gene in transgenic potato plants inhibits viral infection. *Plant Mol Biol* **17,** 431–439

27 Hayakawa, T , Zhu, T , Itoh, K , Kimura, Y , Izawa, T., Shimamoto, K and Toriyama, S. (1992) Genetically engineered rice resistant to rice stripe virus, an insect transmitted virus *Proc Natl Acad Sci USA* **89,** 9865–9869

28 Kaniewski, W K , Lawson, C., Sammons, B , Haley, L , Hart, J , Delannay, X , and Tumer, N E (1990) Field resistance to transgenic Russet Burbank potato to effects of infection by potato virus X and potato virus Y *Biotechnology* **8,** 750–754

29 Farinelli, L , Malnoe, P , and Collet, G F (1992) Heterologous encapsidation of potato virus Y strain O (PVYO) with the transgenic coat protein of PVY strain N (PVYN) in *Solanum tuberosum* CV bintje *Biotechnology* **10,** 1020–1025

30. Kollar, A , Thole, V., Dalmay, T , Salamon, P., and Balazs, E (1993) Efficient pathogen-derived resistance induced by integrated potato virus Y coat protein gene in tobacco *Biochimie* **75,** 623-629

31 Sudarsono, Young, J B , Woloshuk, S L , Perry, D C , Hellmann, G M , Wernsman, E A , Lommel, S A , and Weissinger, A K. (1995) Transgenic burley and fluecured tobacco *(Nicotiana tabacum L )* with resistance to various necrotic isolates of potato virus Y *Phytopathology* **85,** 1493–1499

32. Van der Vlugt, R A A , Ruiter, R K , and Goldbach, R (1992) Evidence for sense RNA-mediated protection to PVY N in tobacco plants transformed with the viral coat protein cistron *Plant Mol Biol* **20,** 631–639

33. Dougherty, W G , Lindbo, J A , Smith, H A , Parks, T. D , Swaney, S , and Proebsting, W M (1994) RNA-mediated virus resistance in transgenic plants exploitation of a cellular pathway possibly involved in RNA degradation *Mol Plant–Microbe Interact* **7,** 544–552

34 Pang, S -Z , Slightom, J L , and Gonsalves, D (1993) Different mechanisms protect transgenic tobacco against tomato spotted wilt and impatiens necrotic spot tospoviruses *Biotechnology* **11,** 819–824.

35 Rezaian, M A , Skene, K G M , and Ellis, J G (1988) Anti-sense RNAs of cucumber mosaic virus in transgenic plants assessed for control of the virus *Plant Mol Biol* **11,** 463–471

36 Powell, P., Stark, D M , Sanders, P R., and Beachy, R N (1989) Protection against tobacco mosaic virus in transgenic plants that express TMV antisense RNA *Proc Natl Acad Sci USA* **86,** 6949–6952

37 Day, A G , Bejarano, E R , Buck, K W , Burrell, M , and Lichtenstein, C P (1991) Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus *Proc Natl Acad Sci USA* **88,** 6721–6725

38 Kawchuk, L. M , Martin, R R , and McPherson, J. (1991) Sense and antisense RNA-mediated resistance to potato leafroll virus in Russet Burbank potato plants *Mol Plant–Microbe Interact* **4,** 247–253

39 Yie, Y., Zhao, F , Zhao, Z., Liu, Y Z , Liu, Y. L , and Tien, P. (1992) High resistance to cucumber mosaic virus conferred by satellite RNA and coat protein in transgenic commercial tobacco cultivar G-140 *Mol Plant–Microbe Interact* **5,** 460–465

40 Huntley, C C and Hall, T C (1993) Minus sense transcripts of brome mosaic virus RNA-3 intercistronic region interfere with viral replication *Virology* **192,** 290–297

41. Nelson, A., Roth, D. A , and Johnson, J D (1993) Tobacco mosaic virus infection of transgenic Nicotiana tabacum plants is inhibited by antisense constructs directed at the 5' region of viral RNA *Gene* **127,** 227–232.

42. Harrison, B D , Mayo, M A , and Baulcombe, D. C (1987) Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA *Nature* **328,** 799–801.
43. Jacquemond, M , Amselem, J , and Tepfer, M. (1988) A gene coding for a monomeric form of cucumber mosaic virus satellite RNA confers tolerance to CMV *Mol Plant–Microbe Interact* **1,** 311–316
44 Gerlach, W. L , Llewellyn, D., and Haseloff, J (1987) Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus *Nature* **328,** 802–805
45. Morch, M. D , Joshi, R L., Denial, T. M , and Haenni, A. L. (1987) A new 'sense' RNA approach to block viral RNA replication in vitro *Nucleic Acids Res* **15,** 4123–4130
46 Stanley, J , Frischmuth, T., and Ellwood, S. (1990) Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. *Proc Natl Acad Sci USA* **87,** 6292–6295
47. Marsh, L. E , Pogue, G P , Connell, J. P., and Hall, T C (1991) Artificial defective interfering RNAs derived from brome mosaic virus *J Gen Virol* **72,** 1787–1792
48 Golemboski, D B., Lomonossoff, G. P , and Zaitlin, M (1990) Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus *Proc Natl Acad Sci USA* **87,** 6311–6315
49 Anderson, J M , Palukaitis, P , and Zaitlin, M (1992) A defective replicase gene induces resistance to cucumber mosaic virus in transgenic tobacco plants *Proc Natl Acad Sci USA* **89,** 8759–8763.
50 Braun, C. J and Hemenway, C L (1992) Expression of amino-terminal portions or full-length viral replicase genes in transgenic plants confers resistance to potato virus X infection. *Plant Cell* **4,** 735–744
51 MacFarlane, S A and Davies, J. W (1992) Plants transformed with a region of the 201-kilodalton replicase gene from pea early browning virus RNA1 are resistant to virus infection *Proc Natl Acad Sci USA* **89,** 5829–5833
52 Rubino, L , Lupo, R , and Russo, M (1993) Resistance to cymbidium ringspot tombusvirus infection in transgenic Nicotiana benthamiana plants expressing a full-length viral replicase gene. *Mol Plant–Microbe Interact* **6,** 729–734.
53. Audy, P , Palukaitis, P , Slack, S. A , and Zaitlin, M. (1994) Replicase-mediated resistance to potato virus Y in transgenic plants. *Mol Plant–Microbe Interact* **7,** 15–22.
54. Brederode, F T , Taschner, P. E. M , Posthumus, E., and Bol, J. F. (1995) Replicase-mediated resistance to alfalfa mosaic virus *Virology* **207,** 467–474
55. Maiti, I. B., Murphy, J. F , Shaw, J G , and Hunt, A. G (1993) Plants that express a potyvirus proteinase gene are resistant to virus infection *Proc Natl Acad Sci USA* **90,** 6110–6114
56 Vardi, E , Sela, I , Edelbaum, O , Livneh, O., Kuznetsova, L , and Stram, Y (1993) Plants transformed with a cistron of a potato virus Y protease (NIa) are resistant to virus infection *Proc Natl Acad Sci USA* **90,** 7513–7517.
57 Beck, D. L., Van Dolleweerd, C J , Lough, T J., Balmori, E , Voot, D M , Andersen, M T , O'Brien, I E W , and Forster, R. L S (1994) Disruption of

virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block *Proc Natl Acad Sci USA* **91,** 10,310–10,314

58 Cooper, B , Lapidot, M , Heick, J A , Dodds, J A , and Beachy, R N. (1995) A defective movement protein of TMV in transgenic plants confers resistance to multiple viruses whereas the functional analog increases susceptibility *Virology* **206,** 307–313

59 Carr, J P and Zaitlin, M (1993) Replicase-mediated resistance *Semin Virol* **4,** 339–347.

60 Fitchen, J H and Beachy, R N (1993) Genetically-engineered protection against viruses in transgenic plants *Annu Rev Microbiol* **47,** 739–763

61 Scholthof, K -B G , Scholthof, H B., and Jackson, A O (1993) Control of plant virus diseases by pathogen-derived resistance in transgenic plants *Plant Physiol* **102,** 7–12.

62. Wilson, T. M A (1993) Strategies to protect crop plants against viruses pathogen-derived resistance blossoms. *Proc Natl Acad Sci USA* **90,** 3134–3141

63 Baulcombe, D C (1994) Replicase-mediated resistance· a novel type of virus resistance in transgenic plants? *Trends Microbiol* **2,** 60–63

64 Turner, N E , Kaniewski, W., Haley, L , Gehrke, L , Lodge, J K , and Sanders, P R (1991) The second amino acid of alfalfa mosaic virus coat protein is critical for coat protein-mediated protection. *Proc Natl Acad Sci USA* **88,** 2331–2335

65 Hill, K K , Jarvis-Eagan, N , Halk, E L , Krahn, K J , Liao, L W , Mathewson, R S , Merlo, D. J., Nelson, S E , Rashka, K. E and Loesch-Fries, L S (1991) The development of virus-resistant alfalfa, *Medicago Sativa L Biotechnology* **9,** 373–377

66 Halk, E L , Merlo, D J , Liao, L W , Jarvis, N. P., and Nelson, S E (1989) Resistance to alfalfa mosaic virus in transgenic tobacco and alfalfa, in *Molecular Biology of Plant-Pathogen Interactions* (Staskawicz, B , Ahlquist, P , and Yolder, O , eds ), Alan R Liss, New York

67 MacKenzie, D. J., Tremaine, J H , and McPherson, J (1991) Genetically engineered resistance to potato virus S in potato cultivar Russett Burbank *Mol Plant–Microbe Interact* **4,** 95–102

68. Namba, S., Ling, K , Gonsalves, C., Gonsalves, D , and Slightom, J. L (1991) Expression of the gene encoding the coat protein of cucumber mosaic virus (CMV) strains WL appears to provide protection to tobacco plants against infection by several different CMV strains. *Gene* **107,** 181–188

69 Okuno, T , Nakayama, M., Yoshida, S., Furusawa, I., and Komiya, T (1993) Comparative suseptibility of transgenic tobacco plants and protoplasts expressing the coat protein gene of cucumber mosaic virus to infection with virions and RNA *Phytopathology* **83,** 542–547

70. Nakajima, M , Hayakawa, T , Nakamura, I., and Suzuki, M (1993) Protection against cucumber mosaic virus (CMV) strains O and Y and chrysanthemum mild mottle virus in transgenic tobacco plants expressing CMV-O coat protein *J Gen Virol* **74,** 319–322.

71 Kallerhoff, J , Perez, P , Bouzoubaa, S , Ben Tahar, S , and Perret, J (1990) Beet necrotic yellow vein virus coat protein-mediated protection on sugarbeet (Beta vulgaris L ) protoplast *Plant Cell Rep* **9,** 224–228

72 Barker, H , Reavy, B., Kumar, A , Webster, K D , and Mayo, M A (1992) Restricted virus multiplication in potatoes transformed with the coat protein gene of potato leafroll luteovirus· similarities with a type of host gene-mediated response *Ann Appl. Biol* **120,** 55–64

73. Bertioli, D J , Cooper, J I., Edwards, M. L., and Hawes, W S (1992) Arabis mosaic nepovirus coat protein in transgenic tobacco lessens disease severity and virus replication *Ann Appl Biol* **120,** 47–54

74. Brault, D J , Candresse, T., le Gall, O., Delbos, R. P., Lanneau, M , and Dunez, J. (1993) Genetically engineered resistance against grapevine chrome mosaic nepovirus *Plant Mol Biol* **21,** 89–97

75 Leclerc, D and AbouHaidar, M G (1995) Transgenic tobacco plants expressing a truncated form of the PAMV capsid protein (CP) gene show CP-mediated resistance to potato aucuba mosaic virus *Mol Plant–Microbe Interact* **8,** 58–65

76 Hoekema, A , Huisman, M J , Molendijk, L , van den Elzen, I P J M , and Cornelissen, B J C (1989) The genetic engineering of two commercial potato cultivars for resistance to potato virus X *Biotechnology* **7,** 273–277

77 Feher, A , Skryabin, K G., Balazs, E., Preiszner, J., Shulga, O A , Zakharyev, V M , and Dudits, D (1992) Expression of PVX coat protein gene under the control of extensin-gene promoter confers virus resistance on transgenic potato plants *Plant Cell Rep* **11,** 48–52

78 Regener, F., da Camara Machado, A , da Camara Machado, M L , Steinkellner, H , Mattanovich, D , Hanzer, H., Weiss, H., and Katinger, H (1992) Coat protein mediated resistance to plum pox virus in Nicotiana clevelandii and N benthamiana. *Plant Cell Rep* **11,** 30–33

79 Fitch, M M. M., Manshardt, R M , Gonsalves, D , Slightom, J. L , and Sanford, J C. (1992) Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. *Biotechnology* **10,** 1466–1472

80 Fang, G. and Grumet, R (1993) Genetic engineering of potyvirus resistance using constructs derived from zucchini yellow mosaic virus coat protein. *Mol Plant–Microbe Interact* **6,** 358–367

81 Sanders, P. R , Sammons, B , Kaniewski, W , Haley, L , Layton, J , LaVallee, B J., Delannay, X., and Tumer, N E. (1992) Field trials of transgenic tomatoes expressing tobacco mosaic or tomato mosaic virus coat proteins *Phytopathology* **82,** 683–690

82 Reimann-Philipp, U and Beachy, R N (1993) Coat protein-mediated resistance in transgenic tobacco expressing the tobacco mosaic virus coat protein from tissue-specific promoters *Mol Plant–Microbe Interact* **6,** 323–330

83. van Dun, C M. P. and Bol, J F (1988) Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus *Virology* **167,** 649–652

84 Ploeg, A. T, Mathis, A , Bol, J F, Brown, D J F, and Robinson, D J (1993) Susceptibility of transgenic tobacco plants expressing tobacco rattle virus coat protein to nematode-transmitted and mechanically inoculated tobacco rattle virus *J Gen Virol* **74,** 2709–2715

85 Gielen, J J L , de Haan, P, Kool, A J , Peters, D , van Grinsven, M Q J M , and Goldbach, R W (1991) Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus *Biotechnology* 1363–1367

86 Mackenzie, D. J. and Ellis, P J (1992) Resistance to tomato spotted wilt virus infection in transgenic tomato expressing the viral nucleocapsid gene *Mol Plant–Microbe Interact* **5,** 34–40

87 Longstaff, M., Brigneti, G., Boccard, F , Chapman, S , and Baucombe, D (1993) Extreme resistance to potato virus X infection in plants expressing a modified component of the putative viral replicase *EMBO J* **12,** 379–386

# 4

# Geminivirus Isolation and DNA Extraction

**Kenneth E. Palmer, Wendelin H. Schnippenkoetter, and Edward P. Rybicki**

## 1. Introduction

Geminiviruses, named for their unique geminate capsid morphology, have small single-stranded (ss) circular DNA genomes that replicate in the nuclei of infected cells via a double stranded (ds) DNA intermediate. They are responsible for economically devastating diseases in a wide variety of crop species from cereals to legumes; it is thus important to gain a better understanding of their epidemiology, genetic diversity, and molecular mechanisms of replication and pathogenicity, for the design of effective resistance strategies (for reviews, *see* **refs.** *1* and *2*). Each geminate particle encapsidates a circular single stranded genomic component of between 2.5 and 3 kb Viruses in the taxonomic family *Geminiviridae* are classified into three genera (Mastrevirus, Curtovirus, Begomovirus), based on their host range, genome organization, and vector species *(3,4)* Mastreviruses, such as maize streak virus (MSV) and wheat dwarf virus (WDV), have monopartite genomes, are transmitted by leafhopper species, and, with a few exceptions, infect monocotyledonous plants. Begomoviruses, such as bean and tomato golden mosaic viruses (BGMV and TGMV), are transmitted by whiteflies (*Bemisia tabacci*) and all infect dicotyledonous plants; most have bipartite genomes, although there are some viruses in this group that apparently have monopartite genomes. Curtoviruses, such as beet curly top and tomato pseudo curly top viruses (BCTV and TPCTV), occupy an intermediate position between Mastreviruses and Begomoviruses, in that these viruses have monopartite genomes and are transmitted by leafhopper species, but only infect dicotyledonous hosts

The genomic organization of geminiviruses is illustrated in **Fig. 1** Geminiviruses rely entirely on the host machinery for replication of the viral genome

From *Methods in Molecular Biology*, Vol 81 *Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

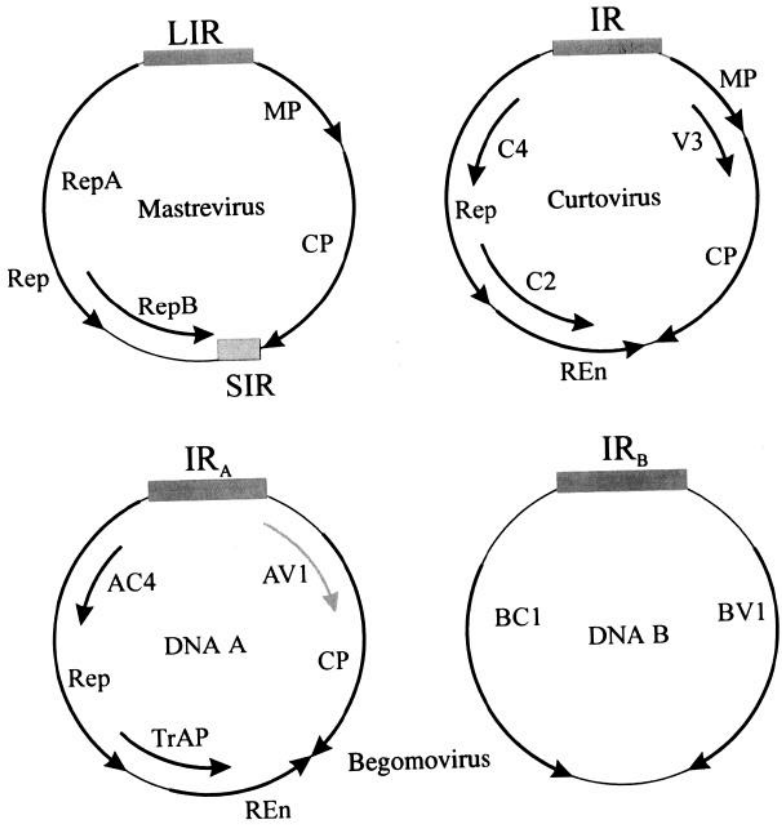



Fig. 1. Genomic organization of geminiviruses. Grey boxes indicate the intergenic regions containing the origin of replication and promoters for bidirectional transcription. The part of the intergenic region whose sequence is identical in both Begomovirus genome components is called the common region (CR). The complementary strand origin of replication in Mastreviruses is in the short intergenic region (SIR). Open reading frames (ORFs) are indicated by black arrows. The convention for naming the ORFs of geminiviruses is that ORFs present in the virion sense of the genome are designated Vx and complementary sense ORFs Cx, where x is a number generally indicating the order in which the ORF occurs. In bipartite geminiviruses, the ORFs are preceded by the letter "A" or "B" to indicate on which genome component the ORF occurs. When a gene's function is known, we have replaced the ORF designation with the gene name. CP, coat protein; MP, movement protein; Rep, replication initiator protein; TrAP, *tr*anscription *a*ctivator *p*rotein; REn, *r*eplication *en*hancer protein ***(5)***. In Curtoviruses, the AC2-encoded protein does not seem to have TrAP activity. The AV1 ORF is only present in Begomoviruses from the Old World; in monopartite Begomoviruses, the AV1 protein may have a function in movement ***(4)***. The proteins encoded by the BV1 and BV2 ORFs are both movement proteins.

and expression of viral genes Specific viral proteins are involved in the initiation of rolling circle replication, *trans*-activation of the CP promoter, production of ssDNA, and viral movement functions (outlined in **Fig. 1**; *see* **refs.** *1* and *2* for comprehensive reviews). Upon entry into the nucleus, host DNA replication machinery converts the virion ssDNA into dsDNA. The double-stranded replicative form DNA (RF-DNA) then functions as both a template for transcription of viral genes and for genome amplification by rolling circle replication, initiated by the Rep protein, which binds specifically to sequences within the intergenic region and induces a nick in the conserved nonanucleotide motif (TAATATT-AC) in the loop of a conserved hairpin-loop structure essential for replication ***(5–9)***. The virion-sense strand released by rolling circle replication circularizes, and is made double-stranded or is encapsidated

Isolation of geminivirus particles can be very difficult: The success of any isolation protocol is highly dependent on the virus and host plant. In this chapter, we describe a simple method developed at the University of Cape Town for isolating MSV virions from infected maize tissue ***(10)***. This employs a low pH extraction buffer and an acidification step, which serves to denature many contaminating plant proteins We then simply subject the clarified sap to two cycles of differential centrifugation, which usually yields pure virus. For geminiviruses that may be more recalcitrant, some authors have found that stirring sap overnight in the presence of Triton X-100 helps to release virus particles from inclusion bodies, prior to two steps of differential centrifugation and purification in sucrose density gradients ***(11,12)***. Alternatively, it may be useful to include a chloroform emulsification step to denature plant proteins before further purification by PEG precipitation and differential centrifugation ***(12,13)***.

Initially, most researchers used virion-associated ssDNA to clone geminivirus genomes: Virions were isolated, ssDNA purified from the virions, and the complementary strand synthesized in vitro This synthesis is simple for Mastreviruses, which have a short DNA primer molecule bound to the virion DNA ***(14–16)***; however, ssDNA from Curto- and Begomoviruses is not usually associated with DNA primers, so second-strand synthesis has to be randomly or specifically primed. The major limitation of this method is that geminivirus virions are frequently difficult, if not impossible, to isolate, and yields are usually low The use of ssDNA as a starting point for molecular manipulations of geminivirus genomes has therefore generally been supplanted by the direct use of viral RF-DNA.

Geminivirus RF-DNA typically accumulates to high levels in the nuclei of infected cells: In our experience, the RF-DNA of MSV is often visible as discrete fast migrating bands in plant DNA extracts electrophoresed in agarose

gels. The RF-DNA in total DNA is amenable to direct manipulation by restriction enzymes; we are able to clone MSV from total DNA extracts quite routinely. It is also easy to enrich total DNA extracts from infected plants for viral RF-DNA by one of several physical or chemical methods developed for the isolation of plasmid DNA, and so treatment of total DNA from infected tissues enriches for the plasmid-like covalently closed circular (ccc) RF-DNA. These techniques include the following:

1 Equilibrium centrifugation in ethidium bromide-CsCl density gradients to separate RF-DNA from genomic DNA *(17)* Ethidium bromide intercalates into DNA and thus confers on cccDNA a different buoyant density to linear or open circular DNA This method requires large amounts of infected tissue, and so may be of somewhat limited practical use
2. The Hirt method *(18)* originally developed for the isolation of polyomavirus DNA from animal cells This method enriches for low-mol-wt DNA by precipitating high-mol-wt DNA in the presence of 1*M* NaCl and 1% SDS, we and others have used it for the isolation of geminivirus replicons from small amounts of callus tissue transfected with recombinant geminivirus constructs (Palmer, Willment, and Rybicki, unpublished results; **ref. *19***) A similar method has found use in the isolation of BGMV RF-DNA *(20)*.
3 Enrichment for cccDNA by denaturation of chromosomal DNA in the presence of alkali *(21–23)*

For isolation of geminivirus RF-DNA, we routinely use the alkaline-lysis plasmid preparation of Ish-Horowitz and Burke *(24)*: This makes use of the observation that there is a narrow range between pH 12 0 and pH 12.5 in which linear, but not cccDNA is denatured We isolate total nucleic acids from infected leaf material by grinding the tissue in liquid nitrogen to break open cells, resuspending the powdered tissue in a DNA-extraction buffer, and extracting with phenol:chloroform, followed by precipitation with isopropanol or ethanol. The nucleic acid pellet is then treated exactly as if it were a plasmid preparation: The high-mol-wt chromosomal DNA is precipitated by alkali treatment and neutralization and is separated from the ccc RF-DNA by centrifugation We also incorporate a further round of purification of RF-DNA by anion-exchange chromatography on commercially available resin columns from a plasmid isolation kit. The protocol for RF-DNA isolation outlined in this chapter therefore yields very clean, highly purified RF-DNA, which is suitable for mapping directly with restriction endonucleases (**Fig. 2**) and even for direct sequencing using specific primers (Rybicki and Wallace, unpublished). We have used it routinely for the isolation of RF-DNA of MSV and the phloem-limited Begomovirus abutilon mosaic virus (Jacobson and Rybicki, unpublished results)

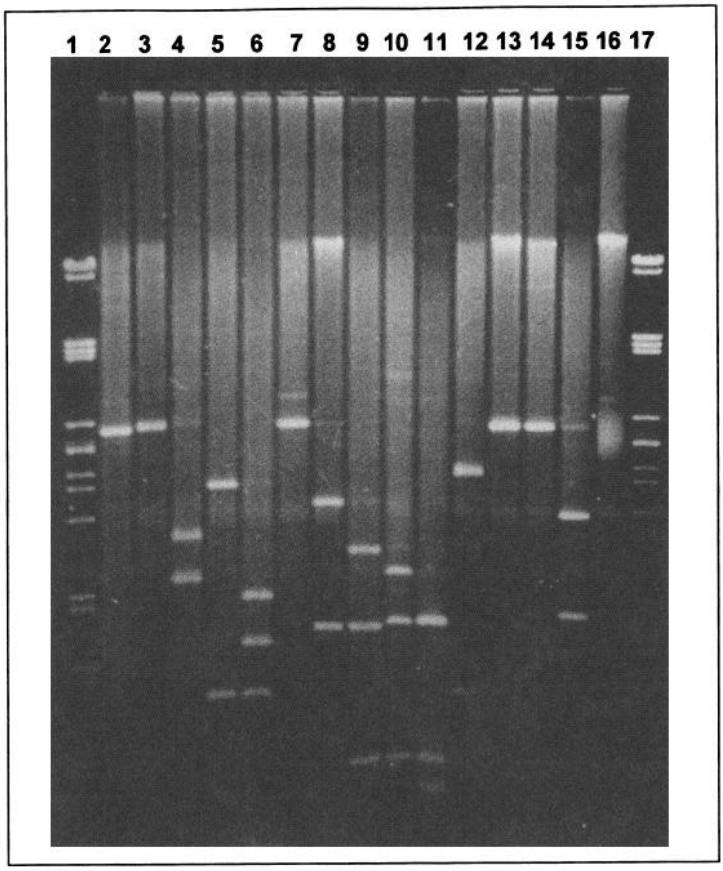



Fig. 2. Restriction endonuclease digestions of the RF-DNA of a severe isolate of MSV from Komatipoort in South Africa. The RF-DNA was purified by the alkaline denaturation–plasmid isolation column method. 300 ng of RF-DNA were used in each restriction digest and electrophoresed in a 0.8% agarose gel, stained with ethidium bromide. Lanes 2–15: lane 2, *Bam*HI; 3, *Bgl*I; 4, *Bam*HI and *Bgl*I; 5, *Hin*dIII; 6, *Hin*dIII and *Bam*HI; 7, *Kpn*I; 8, *Pvu*II; 9, *Pvu*II and *Bam*HI; 10, *Sac*I; 11, *Sac*I and *Bam*HI; 12, *Bgl*I; 13, *Sal*I; 14, *Xho*I; 15, *Xho*I and *Bam*HI; 16, undigested RF-DNA. Lanes 1 and 17, mol-wt marker (λ DNA digested with *Pst*I).

In cases in which only limited amounts, or poor quality, of infected tissue are available, or in which virus DNA accumulates to particularly low levels, we suggest that researchers consider using methods based on the polymerase chain reaction (PCR) to generate large amounts of double stranded virus DNA that are suitable for cloning purposes. Degenerate PCR primers designed to amplify small genomic segments of virtually all Mastreviruses are described

by Rybicki and Hughes *(25)*; similarly, Rojas et al. *(26)* reported sets of primers useful for amplification of parts of the genome of all Curto- and Begomoviruses. If the PCR products are cloned and sequenced, one can then design abutting or partially overlapping PCR primers that will facilitate amplification and cloning of an entire genomic component of the virus of interest (*see* **refs. *27–29***).

## 2. Materials

### 2.1. Isolation of Maize Streak Virus Virions and ssDNA

#### 2.1.1. Isolation of MSV Virions

1 Infected leaf material (about 100 g) (*see* **Note 1**)
2 0 1*M* Sodium acetate buffer pH 4 8 *(30)*
3 0 05*M* Sodium phosphate buffer, pH 7.5
4 Waring-type blender
5 Cheesecloth.
6 Polypropylene centrifuge tubes (Sorvall GSA, or equivalent, Newtown, CT), polycarbonate centrifuge tubes (for Beckman type 35 rotor, or equivalent, Palo Alto, CA)

#### 2.1.2. Isolation of ssDNA from MSV virions

1 DNA extraction buffer 0.1*M* Tris-HCl, 0.1*M* NaCl, 0.1*M* EDTA, pH 7 0, autoclaved. After autoclaving, add SDS to 1% (w/v)
2 Tris-buffered phenol, prepared as described in Sambrook et al *(31)*. Melt solid phenol at 68°C Add 8-hydroxyquinoline to a final concentration of 0 1% Add an equal volume of 0 5*M* Tris-HCl, pH 8 0, and stir the mixture on a magnetic stirrer for 15 min Allow the two phases to separate, then aspirate as much of the upper aqueous phase as possible Add an equal volume of 0 1*M* Tris-HCl to the phenol, stir again, and remove the aqueous phase as before Repeat these extractions until the pH of the phenolic phase is >7.8. Store equilibrated phenol at 4°C for short term storage, or at –20°C. **Caution:** Handle solutions containing phenol with caution, and in a fume hood. phenol is toxic and highly corrosive
3. Chloroform (**Caution:** to be handled in a fume hood, since chloroform is carcinogenic)
4 Sterile 1.5-mL microcentrifuge tubes
5 Sterile TE buffer· 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8 0

### 2.2. Isolation of Total DNA from Infected Plant Material

1 Liquid nitrogen, mortar, and pestle
2 Polypropylene centrifuge tubes (Sorvall SS34, or equivalent), 1 5- and 2 0-mL microcentrifuge tubes (sterilized by autoclaving).
3. DNA extraction buffer: 0 1*M* Tris-HCl, 0.1*M* NaCl, 0 1*M* EDTA, pH 7 0, autoclaved After autoclaving, add SDS to 1% (w/v)

4 Tris-buffered phenol, prepared as described in **Subheading 2.1.2.** (**Caution:** Handle solutions containing phenol with caution, and in a fume hood Phenol is toxic and highly corrosive )
5 Chloroform (**Caution:** to be handled in a fume hood, since chloroform is carcinogenic)
6 Isopropanol (propan-2-ol)
7 Absolute ethanol and 70% ethanol
8 TE buffer 10 m*M* Tris-HCl, 1m*M* EDTA, pH 8 0, sterilized by autoclaving

### 2.3. Isolation of RF-DNA by Alkaline Denaturation and Anion-Exchange Chromatography

1. Commercially available plasmid isolation kit, for example the Qiagen-tip 20 kit (Qiagen GmbH and Qiagen, Hilden, Germany), with Qiagen-tip 20 columns and solutions
   P1· 50 m*M* Tris-HCl, 10m*M* EDTA, pH 8 0, 100 µg Rnase A
   P2 200 m*M* NaOH, 1% SDS
   P3 3 0*M* KOAc, pH 5 5
   QBT 750 m*M* NaCl, 50 m*M* MOPS, 15% ethanol, pH 7 0, 0 15% Triton X-100
   QC 1 0*M* NaCl, 50 m*M* MOPS, 15% ethanol, pH 7 0
   QF 1 25*M* NaCl, 50 m*M* Tris-HCl, 15% ethanol, pH 8 5
2 Sterile 1 5-mL microcentrifuge tubes.
3 Sterile TE buffer 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8 0

## 3. Methods

### 3.1. Isolation of Maize Streak Virus Virions and ssDNA

1 Homogenize freshly harvested leaf material (*see* **Note 2**) from infected plants with an equal weight/volume of 0 1*M* acetate buffer, pH 4 8, at room temperature.
2 Squeeze the homogenate through a layer of cheesecloth
3 Immediately adjust the pH of the homogenate back to pH 4 8 with 10% glacial acetic acid
4 Remove the precipitated plant components by low speed centrifugation (12,000*g* for 10 min in a Sorval GSA rotor)
5 Pellet the virions by ultracentrifugation (130,000*g* for 150 min in a Beckman Type 35 rotor)
6 Resuspend the high-speed pellet in 0 05*M* sodium phosphate buffer, pH 7 5, and repeat **steps 3** and **4**
7 Resuspend the virus pellet in 0 05*M* sodium phosphate buffer, pH 7 5 Alternatively, if the virus is to be used for extraction of ssDNA, resuspend in DNA extraction buffer
8. Isolate ssDNA from virions by extraction with an equal volume of phenol chloroform (1 1) Remove the aqueous phase containing ssDNA to a fresh tube and re-extract with phenol chloroform, if necessary Remove residual phenol by extraction with chloroform

9. Precipitate the viral ssDNA by adding 2.5 vol of ice-cold ethanol. After at least 30 min at –20°C, pellet the precipitated nucleic acid in a microcentrifuge at 4°C. Wash the pellet with 70% ethanol, then air-dry at room temperature. Resuspend the ssDNA in sterile TE buffer.

### 3.2. Extraction of Total Nucleic Acids from Infected Plants

1 Grind 5–10 g of infected leaf material to a fine powder in liquid nitrogen with a mortar and pestle (*see* **Note 3**)
2 Suspend the frozen powdered leaf material in an equal volume of DNA extraction buffer and stir until the mixture reaches room temperature (*see* **Note 4**) If the homogenized plant material is too viscous, add a little more extraction buffer
3. Centrifuge the mixture for 10 min at 4°C in 30-mL polypropylene tubes at 12,000*g* (in a Sorvall SS34 fixed angle rotor) to pellet the plant debris.
4. Transfer the supernatant to 30-mL polyproylene tubes containing 10 mL of Tris-buffered phenol and mix well Centrifuge at 4°C for 10 min at 12,000*g*
5 Transfer the aqueous phase into clean polypropylene tubes (*see* **Note 5**). Add an equal volume of chloroform, mix, and centrifuge again at 4°C for 10 min at 12,000*g* Repeat this step
6 Transfer the aqueous phase to clean polypropylene tubes Add 0 7 vol of isopropanol and mix gently A stringy precipitate of nucleic acids should appear in the tube
7 Pellet the nucleic acids at 17,000*g* for 20 min, discard the supernatant, and stand the tubes upside down to drain off excess isopropanol
8 Wash the pellet by adding about 10 mL of 70% ethanol, dislodge the pellet, and mix and centrifuge again at 17,000*g* for 5 min at 4°C (*see* **Note 6**).
9 Invert the centrifuge tube on absorbent paper and allow the pellet to air-dry for about 10 min at room temperature.
10 If only a crude nucleic acid preparation is required, resuspend the pellet in 0 5–1 mL of TE buffer (*see* **Note 7**)

### 3.3. Isolation of RF-DNA by Alkaline Denaturation and Anion-Exchange Chromatography

This procedure uses a plasmid isolation protocol based on alkaline denaturation of chromosomal DNA and purification of cccDNA by anion-exchange chromatography. The protocol is basically as described by the manufacturers of the kit that we use (the Qiagen-tip 20 kit, Qiagen GmbH and Qiagen), but can easily be adapted for the use of similar plasmid isolation kits.

1 Resuspend the total nucleic acid pellet from **Subheading 3.1., step 9** in 0 3–0 5 mL of solution P1 in a microcentrifuge tube (*see* **Note 8**).
2 Add the same volume of solution P2 Mix gently to avoid shearing chromosomal DNA and incubate at room temperature for 5 min
3 Add the same volume (0 3–0 5 mL) of ice-cold solution P3 Mix well and place on ice for 10 min

4 Pellet the denatured chromosomal DNA by centrifugation at 4°C in a microcentrifuge
5 Decant the supernatant to a clean microcentrifuge tube.
6 Equilibrate the plasmid isolation column by passing 1 mL of solution QBT through the column
7 Add the DNA supernatant to the equilibrated column (*see* **Note 9**).
8. Once the supernatant has passed through the column, wash the column twice with 1 mL of solution QC
9. Elute RF-DNA with 0 5–0.8 mL of solution QF. Collect the eluate in a clean microcentrifuge tube
10 Add 0 7 vol of isopropanol, mix gently, and pellet the DNA in a microcentrifuge for 20 min.
11. Discard the supernatant, and wash the pellet by adding 200 µL of cold 70% ethanol and centrifuging for 10 min at 4°C
12. Discard the supernatant, invert the tube on absorbant paper, and allow the pellet to air-dry
13. Resuspend the RF-DNA in 10–50 µL of TE buffer (*see* **Note 10**)

## 4. Notes

1. It is best to use young leaf material, infected within the previous 2–3 wk We have found that older leaf material usually yields mainly single, not geminate, particles
2 In our experience harvested material may also be stored at 4°C for 2–3 d with no deleterious effects on virus isolation.
3. A standard electric coffee grinder is a good alternative to the mortar and pestle. The plant material is frozen in liquid nitrogen, transferred to the grinding chamber, and processed with short grinding spurts (5–10 s) Before it thaws, transfer the ground material to a beaker containing the DNA-extraction buffer
4 Oxidation of sap components can occur during the extraction procedure, resulting in the DNA pellet being colored yellow to brown This may affect the quality of the DNA extract If oxidation is found to be a problem, we suggest adding 2-mercaptoethanol to the extraction buffer to a final concentration of 10 m*M* (**Caution:** mercaptoethanol is toxic and smells unpleasant, so should be confined to a fume hood )
5 Take care not to disturb the interphase between the aqueous and phenol phases. It will be necessary to repeat the phenol extraction if any denatured protein matter contaminates the aqueous phase.
6. To clean the DNA preparation further, it is sometimes advisable to resuspend the DNA pellet at this stage in 2–4 mL of TE and to reprecipitate the DNA by adding 0.1 vol of 4*M* LiCl and 2 vol of ethanol, and pelleting the DNA again at 4°C This step is not always necessary, and may be omitted if the pellet looks clean
7. We have often found that DNA isolated from maize plants infected with severe isolates of MSV contains such high amounts of RF-DNA that further treatment to enrich for RF-DNA is unnecessary However, plants showing milder symptoms will usually yield less RF-DNA

8 It may be difficult to resuspend large amounts of plant total nucleic acids in such a small volume of buffer P1 If larger volumes are required, scale up the amounts of P2 and P3 used, proportionally
9 A high concentration of residual plant DNA after the alkaline lysis procedure may congest the column To remedy this problem, precipitate the supernatant from **step 5** with 0 8 vol of isopropanol, pellet the DNA in a microcentrifuge and repeat the alkaline denaturation procedure (**steps 1–5**)
10 Trace amounts of contamination with genomic DNA are unavoidable, however, the RF-DNA preparation is usually clean enough for standard molecular manipulations like restriction mapping, cloning, and even direct DNA sequencing

## References

1 Lazarowitz, S G (1992) Geminiviruses· genome structure and gene function *Crit Rev Plant Sci* **11,** 327–349
2 Timmermans, M C P, Das, O P, and Messing, J. (1994) Geminiviruses and their use as extrachromosomal replicons. *Annu Rev Plant Physiol. Plant Mol Biol* **45,** 79–112
3 Briddon, R W and Markham, P G (1995) Geminiviridae, in *Virus Taxonomy Sixth Report of the International Committee on the Taxonomy of Viruses* (Murphy, F A, Fauquet, C M, Bishop, D H L., Ghabrial, S. A., Jarvis, A W, Martelli, G P, Mayo, M. A, and Summers, M D, eds ), Springer-Verlag, Vienna, pp 158–165
4 Rybicki, E P (1994) A phylogenetic and evolutionary justification for three genera of Geminiviridae *Arch Virol* **139,** 49–77
5 Bisaro, D M (1996) Geminivirus replication, in *Eukaryotic DNA Replication* (De Pamphilis, M, ed ), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
6 Laufs, J, Traut, W, Heyraud, F, Matzeit, V, Rogers, S G, Schell, J, and Gronenborn, B (1995) *In vitro* cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus *Proc Natl Acad Sci USA* **92,** 3879–3883
7 Laufs, J, Jupin, I, David, C, Schumacher, S, Heyraud-Nitschke, F, and Gronenborn, B (1995) Geminivirus replication genetic and biochemical characterisation of Rep protein function, a review *Biochimie* **77,** 765–773
8 Stanley, J. (1995) Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle replication *Virology* **206,** 707–712
9 Orozco, B M and Hanley-Bowdoin, L (1996) A DNA structure is required for geminivirus replication origin function *J Virol* **70,** 148–158
10. von Wechmar, M B and Milne, R G (1983) Purification and serology of a South African isolate of maize streak virus, in *Proceedings International Maize Virus Disease Colloquium and Workshop, 2-6 August 1992* (Gordon, D. T, Knoke, J K, Nault, L R, and Ritter, R M, eds ), Ohio State University, Wooster, OH
11 Hamilton, W D O, Saunders, R C, Coutts, R H A, and Buck, K W (1981) Characterisation of tomato golden mosaic virus as a geminivirus *FEMS Microbiol Lett* **11,** 263–267

12 Czosnek, H., Ber, R., Antignus, Y, Cohen, S , Navot, N., and Zamir, D (1988) Isolation of tomato yellow leaf curl virus, a geminivirus *Phytopathology* **78,** 508–512.
13 Sequeira, J C and Harrison, B D (1982) Serological studies on cassava latent virus *Ann Appl Biol* **101,** 33–42.
14 Mullineaux, P M , Donson, J , Morris-Krsinich, B. A M , Boulton, M I , and Davies, J W (1984) The nucleotide sequence of maize sreak virus DNA *EMBO J* **3,** 3063–3068
15 Donson, J , Morris-Krsinisch, B. A. M , Mullineaux, P M , Boulton, M I., and Davies, J W (1984) A putative primer for second-strand synthesis of maize streak virus is virion-associated *EMBO J.* **3,** 3069–3073.
16 Howell, S. H (1984) Physical structure and genetic organisation of the genome of maize streak virus (Kenyan isolate) *Nucleic Acids Res* **12,** 7359–7375
17 Stanley, J and Townsend, R (1985) Characterisation of DNA forms associated with cassava latent virus infection *Nucleic Acids Res* **13,** 2189–2206
18 Hirt, B (1967) Selective extraction of polyoma DNA from infected mouse cell cultures *J Mol Biol* **26,** 365–369
19. Xie, Q., Suárez-López, P , and Gutiérrez, C (1995) Identification and analysis of a retinoblastoma binding motif in the replication protein of a plant DNA virus requirement for efficient viral DNA replication *EMBO J* **14,** 4073–4082
20 Gilbertson, R L , Faria, J C , Hanson, S F , Morlaes, F. J., Ahlquist, P , Maxwell, D. P., and Russel, D. R. (1991) Cloning of the complete genomes of four bean-infecting geminiviruses and determining their infectivity by electric discharge particle acceleration *Phytopathology* **81,** 980–985
21 Sunter, G , Coutts, R H A , and Buck, K W (1984) Negatively supercoiled DNA from plants infected with a single stranded DNA virus *Biochem Biophys Res Commun* **118,** 747–752
22 Srivistava, K M., Hallan, V , Raizada, R K , Chandra, G., Singh, B P , and Sane, P V (1995) Molecular cloning of Indian tomato leaf curl virus following a simple method of concentrating the supercoiled replicative form of viral DNA *J Virol Methods* **51,** 297–304
23 Bendahmane, M., Schalk, H -J., and Gronenborn, B. (1995) Identification and characterisation of wheat dwarf virus from France using a rapid method for geminivirus DNA preparation. *Phytopathology* **85,** 1449–1455
24. Ish-Horowitz, D and Burke, J F (1982) Rapid and efficient cosmid vector cloning *Nucleic Acids Res* **9,** 2989–2998.
25. Rybicki, E P and Hughes, F. L (1990) Detection and typing of maize streak virus and other distantly related viruses of grasses by polymerase chain reaction amplification of a conserved viral sequence. *J Gen. Virol* **71,** 2519–2526
26 Rojas, M. R., Gilbertson, R L , Russel, D R , and Maxwell, D P (1993) Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses *Plant Disease* **77,** 340–347
27 Briddon, R. W , Prescott, A. G , Luness, P., Chamberlin, L C. L., and Markham, P G. (1993) Rapid production of full-length, infectious geminivirus clones by abutting primer PCR (AbP-PCR) *J Virol Methods* **43,** 7–20

28 Patel, V P, Rojas, M R, Paplomatas, E. J., and Gilbertson, R L (1993) Cloning biologically active geminivirus DNA using PCR and partially overlapping primers *Nucleic Acids Res* **21,** 1325–1326

29 Tan, P H N, Wong, S M, Wu, M, Bedford, I D, Saunders, K, and Stanley, J (1995) Genome organisation of ageratum yellow vein virus, a monopartite whitefly-transmitted geminivirus isolated from a common weed *J Gen Virol* **76,** 2915–2922

30 Miller, G. L and Golder, R H (1950) Buffers of pH 2 to 12 for use in electrophoresis. *Arch Biochem* **29,** 420–423.

31 Sambrook, J Fritsch, E F, and Maniatis, T (1989) *Molecular Cloning A Laboratory Manual*, 2nd ed Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

# 5

# Caulimovirus Isolation and DNA Extraction

**Simon N. Covey, Rob J. Noad, Nadia S. Al-Kaff, and David S. Turner**

## 1. Introduction

Members of the caulimovirus group *(1)* each have a circular double-stranded DNA genome of approx 8 kbp that is encapsidated in a spherical, naked nucleocapsid of approx 50 nm diameter (**Fig. 1**). Caulimoviruses characteristically produce subcellular inclusion bodies in infected tissues that contain most of the virions found in cells, embedded in an apparently random manner. The host ranges of individual caulimoviruses tend to be restricted to one or a few plant families, and group members are transmitted between plants by aphid vectors Based on possession of all, or most, of these characteristics, 12 definite, and 3 possible, members of the group have been identified *(2)*

The best-characterized and type member of the caulimoviruses is cauliflower mosaic virus (CaMV), from which the group name derives. The complete nucleotide sequence of at least eight different CaMV isolates *(3–10)*, and that of four other caulimoviruses *(11–14)* has been determined. The organization of viral genes (**Fig. 1**) is mostly conserved in sequenced caulimoviruses, but one member, cassava vein mosaic virus (CVMV), is somewhat different from the others *(14)*. Replication of caulimoviruses involves alternation of genomes as DNA and RNA forms, progeny virion DNA being generated by reverse transcription of a terminally redundant, genome-length RNA utilizing a virus-encoded polymerase This feature is shared by another group of plant DNA viruses, the badnaviruses, and by animal hepatitis B viruses. Such viruses have been termed pararetroviruses to distinguish them from animal retroviruses, which package an RNA form of the genome derived by transcription of an integrated provirus. Sequence homologies in putative coding regions of different caulimoviruses are relatively low One short sequence is conserved among

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

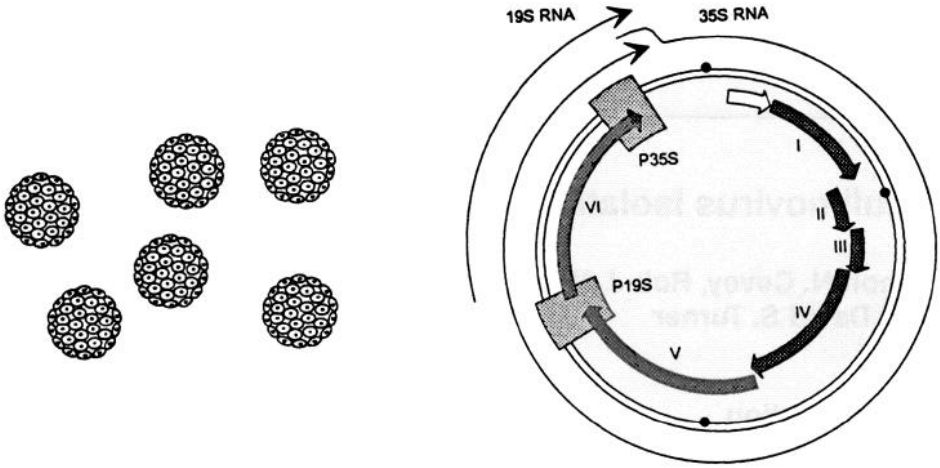



Fig. 1. Virions and genome organization of a typical caulimovirus, CaMV. The virus particles of CaMV (left) are isometric and about 50 nm in diameter (the subunits are schematic and are not a true representation). The DNA genome of CaMV is a circular double-stranded DNA of 8 kbp with three site-specific discontinuities (small closed circles). One of these (top of map) is in the DNA (–)-strand and is adjacent to the sequence homologous to the host tRNA that primes CaMV DNA synthesis by reverse transcription of 35S RNA. The other two gaps are in the (+)-strand adjacent to sequences controlling initiation of (+)-strand DNA synthesis. The genome has six major open reading frames (inner closed arrows), for which protein products have been identified. Gene I encodes a protein involved in cell-to-cell spread, gene II specifies the aphid transmission factor, the gene III product is a DNA-binding protein associated with virions, gene IV encodes the major CP, gene V specifies the viral polymerase (reverse transcriptase and RNAse H), and the gene VI product is an apparently multifunctional protein involved in transactivating viral protein synthesis, and in sequestering virions in inclusion bodies; it is also a major pathogenic determinant of symptom development. There are two major viral transcripts: 35S RNA, which probably serves two roles, one as a replication template and another as a viral mRNA; and 19S and 35S promoters (P19 and P35), respectively.

all members of the group comprising a 13- to 16-base element complementary to the 3' end of host methionyl initiator tRNA at the origin of viral replication. In fact, this sequence seems to be conserved in most plant genetic elements utilizing a reverse transcription strategy. Other regions of homology among caulimoviruses reside in parts of the CP and polymerase genes.

Symptoms produced in plants by CaMV infections vary, depending on the virus isolate and host species. Typical CaMV isolates in highly susceptible hosts, such as *Brassica rapa* (e.g., turnip), cause local lesions when inoculated

onto the second leaf of 10- to 14-d-old seedlings by 4–7 d postinoculation (pi). Systemic symptoms appear as vein clearing, first, in a sector of leaf 3 or 4 by about 10–12 d pi, and then covering the whole leaf of all subsequent leaves to emerge. Vein clearing develops into vein banding by about 20 d pi, and uniform leaf chlorosis by 30 d pi. Leaves also develop as distorted, stunted structures. Different isolates of CaMV cause a range of variations on this theme from mild to severe symptoms. However, other *Brassica* hosts (e g., *B oleraceae* variants) develop markedly less severe symptoms, although *Arabidopsis* is one of the more susceptible host species.

Of those caulimoviruses that have been purified, the method used has been essentially similar to that originally described for CaMV ***(15)***, and upon which the method described here is based. The main problem to overcome in purifying caulimoviruses from infected tissue is disruption of the inclusion bodies containing the virions. In fact, two types of inclusion body have been identified in CaMV-infected tissues. The electron-lucent inclusion bodies, with a matrix consisting of the protein product of CaMV gene II and involved in aphid acquisition ***(16)***, contain relatively few virions. In contrast, the electron-dense inclusion bodies, with a matrix consisting of the viral gene VI product, contain most of the cellular virions ***(17,18)***. The CaMV virion itself is fairly robust and, depending on the isolate, can, under certain circumstances, withstand the rigors of incubation in phenol without releasing its DNA ***(19)***. The virion isolation method described here was designed to liberate virions from inclusion bodies by incubation of plant extracts in urea ***(15)*** Solublization of cells and prevention of virus aggregation is enhanced by inclusion of the nonionic detergent Triton X-100, and polyphenoloxidase activity is minimized by the reducing agent sodium sulphite Liberated virions are purified by differential centrifugation. Virion DNA is released from purified virus by digestion with proteinase K In the following protocols, we have included our standard method of CaMV isolation, which is based on a longer procedure originally described by Hull et al ***(15)***, and a quick method for isolating CaMV DNA from smaller amounts of tissue. A further rapid method of isolating high yields of CaMV DNA has been described by Gardner and Shepherd ***(20)***. We also include description of a two-dimensional (2D) gel electrophoresis method we have used to study complex DNA populations, which has allowed us to characterize replicative forms of caulimoviral DNA ***(21,22)***.

## 2. Materials

### 2.1. Virion Purification

1 Infectious inoculum Sap made by grinding 1 $cm^2$ infected leaf in 1 mL sterile water, purified virus, purified virion DNA, cloned virion DNA in TE (10 m*M* Tris-HCl, pH 7.8, 1 m*M* EDTA).
2 Celite abrasive.

3. Sterilized glass rod with tip flattened obliquely
4. Sodium phosphate buffer, pH 7 2 Stock solutions, 0 5*M* $Na_2HPO_4$ (17 91 g/100 mL), 0 5*M* $NaH_2PO_4$ (7 8 g/100 mL) For 200 mL of buffer, mix 144 mL of 0 5*M* $Na_2HPO_4$ with 56 mL 0 5*M* $NaH_2PO_4$, cool to 4°C
5 Solid sodium sulfite (0 75%)· Weigh out 1 5 g per 200 mL extraction buffer
6 Solid urea (1*M*). Weigh out 12 g for 200 mL buffer
7. 10% Triton X-100 (stock solution)
8. DNase buffer. 50 m*M* Tris-HCl, pH 7 5, 5 m*M* $MgCl_2$
9. Phenol:chloroform mixture· phenol:chloroform·isoamyl alcohol (25.24.1). **Caution:** Phenol·chloroform is extremely corrosive and toxic. It is best bought as a preprepared solution; handle in small volumes with great care
10 10 mg/mL Proteinase K (Boehringer) in TE with 1% SDS
11. 2 mg/mL Deoxyribonuclease I (DNase I, Sigma) in DNase buffer
12 2 mg/mL Pancreatic ribonuclease A (RNase A) in TE (heat-treated by incubation at 95°C for 10 min)
13 10% Sodium dodecyl sulfate (SDS)
14 TE solution
15. 0 5*M* $MgCl_2$
16 30% Polyethylene glycol 6000 (PEG)
17 Centrifuge rotors cooled to 4°C. Sorvall GSA high speed rotor (6 × 500 mL), Sorvall TFT 65.38 (8 × 38 mL), Sorvall TFT 65 13 (12 × 13 mL) or equivalent rotors
18 Bottom-drive blender cooled to 4°C
19 Muslin (four layers), washed in distilled water and squeezed dry
20 Rubber policeman (round-ended glass rod covered at one end with a rubber sleeve)

### 2.2. 2D Gel Electrophoresis

1 Agarose
2 Neutral dimension buffer (TA). 25 m*M* Tris-acetate, pH 7.9
3 Alkaline solution for second denaturing dimension 30 m*M* NaOH, 2 m*M* EDTA
4 Tracking dye. 1% orange G, 20% Ficoll, 5 m*M* EDTA in appropriate running buffer
5. Depurination solution 100 m*M* HCl
6 Denaturing solution 0 5*M* NaOH, 1 5*M* NaCl.
7 Neutralizing solution 1*M* Tris-HCl, pH 7 6, 1 5*M* NaCl
8 Transfer solution 3*M* NaCl, 0.3*M* trisodium citrate

## 3. Methods

### 3.1. Inoculation of Plants

1. Inoculum should contain one of the following in 10 µL of solution. infectious sap in water, 0 1–1 0 µg purified virions in water, 1–2 µg purified virion DNA in TE, 2–4 µg cloned virion DNA in TE treated with the appropriate restriction enzyme to liberate the viral DNA from the cloning vector. Cloned CaMV DNA is infectious when inoculated as linear molecules in a mixture with cloning vector DNA
2 Add a trace of celite abrasive to the solution and apply 10 µL per plant on the second true leaf when plants are at the two-leaf stage, but with the inoculated leaf

not yet fully expanded. Gently stroke the glass rod about six times, pressing the liquid lightly over the leaf surface.

3 Propagate plants at 16–20°C in a 16-h photoperiod For maximal virus yields, harvest between about 15–25 d pi

### 3.2. Large-Scale Virus Purification

1 Harvest 100 g turnip leaves, systemically infected with CaMV Select younger leaves up to ca. 10 cm in length Wash and lightly dry leaves (we use a salad centrifuge for this).
2. Place leaves in the blender with 200 mL 0 5*M* sodium phosphate buffer, pH 7 2, and 0 75 g sodium sulphite per 100 mL homogenate. Blend to fine fragments in the cold
3 Pour homogenate into a beaker, adding 6 g urea and 25 mL of 10% Triton X-100, both per 100 mL homogenate Stir with magnetic stirrer at 4°C overnight
4 Centrifuge extracted homogenate at 4000*g* for 10 min at 4°C in an appropriate high-speed rotor
5 Gently pour the supernatant through four layers of muslin and then distribute the green liquid into tubes for pelleting virus by ultracentrifugation at 70,000*g* for 2 h at 4°C in an ultracentrifuge
6 Pour off supernatant and resuspend pellets (pellets might be slightly green and also contain starch as well as virus) by dispersing in 1 mL sterile distilled water (SDW) per tube for 1–2 h using a rubber policeman occasionally
7 Pool resuspended pellets and centrifuge twice in a microcentrifuge to remove particulate matter
8 Pellet virions from the supernatant (volumes can be made up with SDW) by ultracentrifugation at 136,000*g* at 4°C for 1 h
9 Resuspend pellets each in 1 mL of DNase buffer. Virion yield can be assessed at this point by UV spectrophotometry A suspension of CaMV virions of 1 mg/mL has an $OD_{260}$ of 7 (adjusted for light scattering). Virions can be stored at 4°C or –20°C
10 To isolate virion DNA, the purified virions are first treated with DNase I (10 µg/mL for 10 min at 37°C, reaction stopped by addition of EDTA to 1 m*M*) to remove fragments of plant DNA. Virions are disrupted by adding stock proteinase K to a final concentration of 0 5 mg/mL with 1% SDS in TE and incubating for 15 min at 37°C. DNA is purified from the lysed mixture by phenol·chloroform extraction (minimum of twice) DNA is concentrated by ethanol-precipitation, collected, and quantified by absorbance at 260 nm.

### 3.3. Quick Method

This method is suitable for isolating CaMV DNA very rapidly from small quantities of tissue when yield is not of major importance and is suitable for PCR analysis, cloning, or sequencing.

1 Grind a single infected leaf in a mortar with 1 mL SDW, add Triton X-100 to 2%, and vortex thoroughly
2. Pellet inclusion bodies containing virus by spinning in a microcentrifuge for 2 min Resuspend pellet in 1 mL SDW and pellet again Repeat spin and wash a total of three times to remove all traces of Triton X-100.

3 Resuspend pellet in 0 8 mL DNase buffer and incubate with 10 μg/mL DNase I and 10 μg/mL pancreatic ribonuclease A at 37°C for 60 min Add SDS to a final concentration of 0 5% (w/v) and incubate at 65°C for 10 min Add proteinase K to 0 5 mg/mL and incubate at 65°C for a further 15 min
4 Spin out debris in microcentrifuge for 3 min. Add magnesium chloride ($MgCl_2$) to 100 m*M* and spin out any precipitate that forms Remove supernatant to a fresh tube and extract with 0 5 mL phenol/chloroform Microcentrifuge for 5 min Remove aqueous supernatant to a fresh tube
5 To selectively precipitate intact viral DNA, excluding fragmented DNA, add $MgCl_2$ to 100 m*M* and 0 6 mL 30% PEG and incubate at room temperature for 10 min Pellet precipitate in microcentrifuge for 5 min Discard supernatant and take up pellet in 0.4 mL SDW and add $MgCl_2$ to 100 m*M* Precipitate DNA by addition of an equal volume of isopropanol, mix, and pellet precipitate Wash DNA precipitate two to three times with 70% ethanol to remove traces of PEG

### 3.4. 2D Gel Electrophoresis of Caulimoviral DNA

1 For a 20 × 20 × 0 45 cm 1% agarose gel, melt 1 8 g agarose in 180 mL TA buffer and pour gel The sample well is formed by placing a 2-mm diameter sealed Pasteur pipet or custom-made well former in a horizontal position about 1–2 cm in from each side of the gel at one of its corners. The well former should not quite touch the glass plate on which the gel is cast
2. Assemble the set gel into its running apparatus Pour on neutral buffer (TA) to submerge the gel totally Set up a buffer recirculation system
3 Prepare DNA sample in a total volume of less than 5 μL containing 0 5–1 μL of tracking dye If the sample is purified virion DNA to be detected by hybridization with a radioactive probe, then less than 20 ng should be loaded If the sample is from a total cellular DNA preparation from infected plants, about 10 μg is loaded
4 Electrophoresis in the first dimension is at 1 25 V/cm for about 24 h
5. To prepare the gel for the second (denaturing) dimension, carefully remove the gel from the running apparatus (it is best to keep the gel on the glass plate on which it was cast) and place in alkaline running medium for 15 min, with gentle agitation, then into fresh alkaline medium for a further 30 min to complete the denaturation
6 Reassemble the gel into the electrophoresis apparatus, being careful to reorientate it at 90° relative to the first dimension Pour denaturing medium into the apparatus and recirculate as before. Size markers can then be loaded into the sample well before denaturing electrophoresis
7 Electrophoresis in the second (denaturing) dimension is at 1 25 V/cm for about 24 h
8. After electrophoresis, in preparation for Southern blotting, the gel is washed for 5 min in distilled water
9. DNA in the gel is depurinated to prevent snap-back of supercoiled or hairpin species. Soak the gel for exactly 10 min in 100 m*M* HCl Wash briefly in distilled water

10 DNA in the gel is then denatured, neutralized, and transferred to nitrocellulose for conventional Southern blot hybridization (*see* Chapter 43).

## 4. Notes

1. In their original isolation method, Hull et al. ***(15)*** reported CaMV yields of 0 6–1 mg virus per 100 g tissue Using a more rapid method of purification from relatively large amounts of leaves, in which virus inclusion bodies were pelleted before they were disrupted with urea and Triton X-100, Hull et al ***(15)*** reported virus yields of 70% of their original method. Our standard method typically yields 1 5–2 mg virus per 100 g tissue We have found that higher yields are generally obtained from younger leaves Greater virus yields (2–4 mg/100 g tissue) have been reported for the rapid CaMV purification method of Gardner and Shepherd ***(20)*** suitable for use with small amounts of tissue Our quick method yields only 0 1 mg virus/100 g tissue, but is most suitable for rapid preparation of viral DNA from large numbers of samples of small amounts of tissue for DNA restriction analysis, PCR, sequencing, or cloning Further virion purification can be achieved by ultracentrifugation through a sucrose gradient, as described by Hull et al. ***(15)***
2 Purification of all other caulimoviruses should be possible using the methods described herein, employing urea to disaggregate the inclusion bodies, which are a characteristic feature of the group
3 CaMV particles have shown resistance to disruption by phenol, and this feature can be exploited to specifically isolate nonencapsidated viral DNA and RNA by phenol chloroform extraction of whole-cell nucleic acid ***(19)*** The resistance to phenol might be explained in part by occlusion of virions in inclusion bodies, which are probably pelleted during centrifugation to separate the phenol.chloroform from the aqueous phase We have heard that some CaMV strains do not show such resistance to phenol, possibly because they are not retained so tightly in inclusion bodies
4 The CP of CaMV is glycosylated ***(23)*** and phosphorylated ***(24)***, and synthesis of the mature CP polypeptide proceeds via a processing step However, analysis of the composition of CaMV CP has been hampered, because during virus purification, the CP can undergo degradation into specific fragments It can also aggregate to form multimeric polypeptides, as resolved on denaturing PAGE ***(25)***.
5 CaMV virion DNA can be identified by its characteristic mobility during gel electrophoresis (**Fig. 2**) Under nondenaturing conditions, virion DNA separates as a genome-length (8 kbp) linear form produced by breakage of the circular DNA, together with more slowly migrating open-circular components The most rapidly migrating open-circular form is of typical open conformation (**Fig. 2**, ND). However, a series of more slowly migrating forms are also observed, which comprise molecules that are twisted to varying degrees (not supercoiled, because the DNA has single-strand discontinuities) The single-stranded components of virion DNA can be revealed by denaturing the sample before electrophoresis (**Fig. 2D**).
6. 2D gel electrophoresis (**Fig. 3**) is an extremely powerful method of resolving complex populations of DNA It allows separation of linear single-stranded and

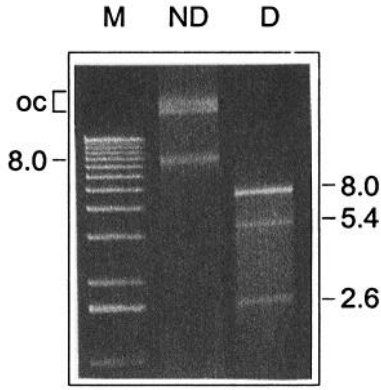
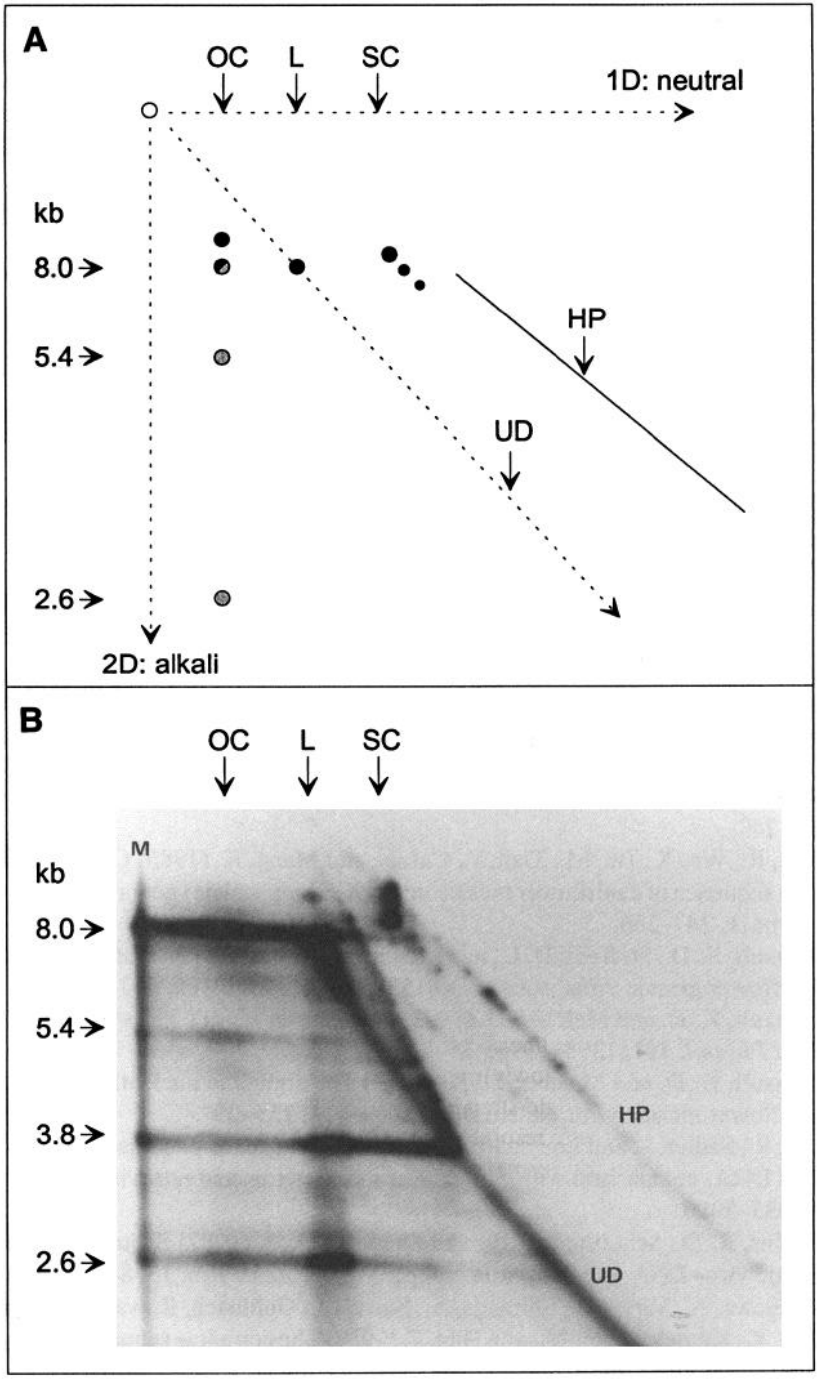



Fig. 2. Gel electrophoresis of caulimovirus virion DNA. DNA from purified CaMV virions was electrophoresed in a nondenaturing minigel and stained with ethidium bromide. M, size markers; ND, nondenatured virion DNA showing typical components resolved as an 8 kbp (left) linear, and open circular (oc) forms, including twisted molecules. D, shows the same DNA as in ND, but denatured by incubation in 50 m*M* NaOH at 65° for 10 min prior to loading on the gel. This shows the typical pattern of three single-stranded DNA components of virion DNA with sizes of 8 kb, 5.4 kb, and 2.6 kb. Note that, since these forms are migrating as single-stranded DNAs in a nondenaturing medium, they do not run with mobility consistent with their sizes relative to the double-stranded DNA size markers (M).

Fig. 3. *(opposite page)* 2D gel electrophoresis of CaMV intracellular DNA. Total cellular DNA was isolated from infected plants by phenol:chloroform extraction, under which conditions most of the cellular virion DNA is not purified. The sample thus contains viral replicative forms and minichromosome (supercoiled) DNA. This complex population of molecules can be readily resolved on 2D gels. In the first (nondenaturing) dimension, DNAs are separated according to size and conformation. Then, in the second (denaturing) dimension at 90° orientation relative to the first, molecules are resolved largely according to single-stranded size. Theoretical migration of various forms is shown in the upper diagram. The sample well is to the top left. Molecules with equivalent relative mobility in both dimensions migrate along a line we call the unit diagonal (UD). Double-stranded linear molecules (L) fall on this line according to size. Open circular molecules (OC) with linear components resolve as slowly migrating forms in the neutral dimension, but are separated into their various single-stranded forms in the second dimension. For instance, conventional OC molecules are resolved into an 8 kb linear and a more slowly migrating closed single-stranded circular form (filled OC spots); CaMV virion DNA is resolved into the three single-stranded components (half-tone OC spots), as described in the legend to **Fig. 2**. Supercoiled (SC) forms migrate more rapidly in the first dimension. Smaller SC molecules form a diagonal to the lower right of the main SC DNA. Hairpin (HP) molecules migrate as double-stranded linear forms in the first dimension, but at half their double-stranded mobility when denatured, since they melt to form single-stranded molecules of twice the double-stranded size. Native single-stranded forms migrate along a diagonal between the UD and the HP diagonal. The lower part of the figure shows an actual separation of CaMV DNA forms. For further explanation of 2D gels and their interpretation, *see* **refs. *21*** and ***22***.


A
OC
L
SC
1D: neutral
kb
8.0
5.4
2.6
HP
UD
2D: alkali
B
OC
L
SC
M
kb
8.0
5.4
3.8
2.6
HP
UD

double-stranded DNAs, discontinuous double-stranded DNAs, open circular, supercoiled, and hairpin molecules *(21,22)*. After electrophoresis and before blotting, a depurination step is included to introduce breaks into molecules, which would otherwise renature by snapback on neutralization The duration/acid concentration of the treatment is fairly critical and will depend upon gel thickness A balance has to be attained between undertreatment, resulting in underrepresentation of snapback forms on the final autoradiogram, and overtreatment, causing a general reduction in hybridization signal of all forms

## References


1 Howell, S H (1985) The molecular biology of plant DNA viruses *CRC Crit Rev Plant Sci* **2,** 287–316

2 Covey, S N and Hull, R (1992) Genetic engineering with double-stranded DNA viruses, in *Genetic Engineering with Plant Viruses* (Davies, J W and Wilson, T M A , eds ), CRC, Boca Raton, p 217

3 Franck, A , Guilley, H , Jonard, G , Richards, K , and Hirth, L (1980) Nucleotide sequence of cauliflower mosaic virus DNA *Cell* **21,** 285–294

4 Gardner, R C , Howarth, A J , Hahn, P , Brownluedi, M , Shepherd, R J , and Messing, J (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. *Nucleic Acids Res* **9,** 2871–2888

5 Howarth, A J , Gardner, R C , Messing, J , and Shepherd, R J (1981) Nucleotide sequence of naturally occurring deletion mutants of cauliflower mosaic virus is transmitted naturally by aphids *Virology* **112,** 678–685

6 Balazs, E , Guilley, H , Jonard, G , and Richards, K (1982) Nucleotide sequence of DNA from an altered-virulence isolate D/H of cauliflower mosaic virus *Gene* **19,** 239–249

7 Fang, R , Wu, X , Bu, M , Tian, Y , Cai, F , and Mang, K (1985) Complete nucleotide sequence of cauliflower mosaic virus (Xinjiang isolate) genomic DNA *Chin J Virol* **1,** 247–256

8 Chenault, K D , Steffens, D. L., and Melcher, U K (1992) Nucleotide sequence of cauliflower mosaic virus isolate NY8153 *Plant Physiol* **100,** 542–545

9 Chenault, K D. and Melcher, U (1993) Cauliflower mosaic virus isolate CMV-1 *Plant Physiol* **101,** 1395–1396

10 Chenault, K D. and Melcher, U K (1993) The complete nucleotide sequence of cauliflower mosaic virus isolate BBC *Gene* **123,** 255–257

11 Hull, R , Sadler, J , and Longstaff, M (1986) The sequence of carnation etched ring virus DNA comparison with cauliflower mosaic virus and retroviruses *EMBO J* **5,** 3083–3090

12 Richins, R. D., Scholthof, H. B., and Shepherd, R J. (1987) Sequence of figwort mosaic virus DNA (caulimovirus group). *Nucleic Acids Res* **15,** 8451–8466

13. Hasegawa, A., Verver, J , Shimada, A , Saito, M , Goldbach, R , van Kammen, A , Miki, K , Kameyaiwaki, M., and Hibi, T (1989) The complete sequence of soybean chlorotic mottle virus DNA and the identification of a novel promoter *Nucleic Acids Res* **17,** 9993–10,015

14 Calvert, L A , Ospina, M D , and Shepherd, R J (1995) Characterization of cassava vein mosaic virus a distinct plant pararetrovirus *J Gen Virol* **76,** 1271–1276
15 Hull, R , Shepherd, R J , and Harvey, J. D (1976) Cauliflower mosaic virus an improved purification procedure and some properties of the virus particle *J Gen Virol* **31,** 93–100
16 Espinoza, A M , Medina, V , Hull, R , and Markham, P G (1991) Cauliflower mosaic virus gene II product forms distinct inclusion bodies in infected plant cells *Virology* **185,** 337–344
17 Shepherd, R J , Richins, R , and Shalla, T A (1980) Isolation and properties of the inclusion bodies of cauliflower mosaic virus *Virology* **102,** 389–400
18 Stratford, R , Plaskitt, K A , Turner, D S , Markham, P G , and Covey, S N (1988) Molecular properties of Bari 1, a mild strain of cauliflower mosaic virus *J Gen Virol* **69,** 2375–2386
19 Hull, R and Covey, S N (1983) Characterisation of cauliflower mosaic virus DNA forms isolated from infected turnip leaves *Nucleic Acids Res* **11,** 1881–1895
20 Gardner, R C and Shepherd, R J. (1980) A procedure for rapid isolation and analysis of cauliflower mosaic virus DNA *Virology* **106,** 159–161
21 Covey, S. N , Turner, D S , Lucy, A. P., and Saunders, K (1990) Host regulation of the cauliflower mosaic virus multiplication cycle *Proc Natl Acad Sci USA* **87,** 1633–1637
22 Turner, D S and Covey, S N (1993) Reverse transcription products generated by defective plus-strand synthesis during cauliflower mosaic virus replication. *Virus Res* **28,** 171–185
23 DuPlessis, D H and Smith, P (1981) Glycosylation of the cauliflower mosaic virus capsid polypeptide *Virology* **109,** 403–408
24. Hahn, P and Shepherd, R J (1982) Evidence for a 58-kilodalton polypeptide as precursor of the coat protein of cauliflower mosaic virus *Virology* **116,** 480–488
25 Al Ani, R , Pfeiffer, P , and Lebeurier, G. (1979) The structure of cauliflower mosaic virus II Identity and location of the viral polypeptides *Virology* **93,** 188–197
26. Al-Kaff, N. S and Covey, S N (1995) Biological diversity of cauliflower mosaic virus isolates expressed in two *Brassica* species *Plant Pathol* **44,** 516–526

# 6

# Reovirus Isolation and RNA Extraction

**Ichiro Uyeda, Bong-Choon Lee, Yuko Ando, Haruhisa Suga, Yun-Kun He, and Masamichi Isogai**

## 1. Introduction

Plant reoviruses are classified in three genera in the family *Reoviridae*: *Phytoreovirus*, *Fijivirus*, and *Oryzavirus*. Fifteen viruses, including possible members, are described *(1)* With two exceptions, all of them infect plants in *gramineae*. They possess 10–12 segmented double-stranded RNAs (dsRNAs) as a genome and are transmitted propagatively by leafhoppers or planthoppers. Rice dwarf *Phytoreovirus* (RDV) is the only plant reovirus whose complete nucleotide sequence is known *(2)*. Rice dwarf virus has 12 genomic segments separated by PAGE. They are numbered from S1 to S12, from the slowest migrating segment, and all the structural and nonstructural proteins of RDV have been assigned *(3)* (**Fig. 1**). Genome characterization of other plant reoviruses is reviewed by Uyeda et al *(4)*.

Although methods described in this chapter are mostly for RDV, those for virus isolation and genome extraction should be applicable to other viruses, since most plant reoviruses have gramineae hosts, and all of them have dsRNAs as a genome. However, purification of the virus particles must be carefully chosen and there seems to be no universal or general methods. We describe those for RDV, rice black-streaked dwarf virus (RBSDV), and rice ragged-stunt virus (RRSV). The most difficult part of the plant reovirus study is to obtain good plant material to start with. The best plant material is fresh and young infected plants grown under an appropriate greenhouse or growth chamber condition. In order to do so, one has to maintain the virus culture by frequent transfers through the vector insect, because they often lose vector transmissibility after prolonged culturing in a plant host Field-grown plant material contains a genomically heterogeneous population of viruses *(5)* and a

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

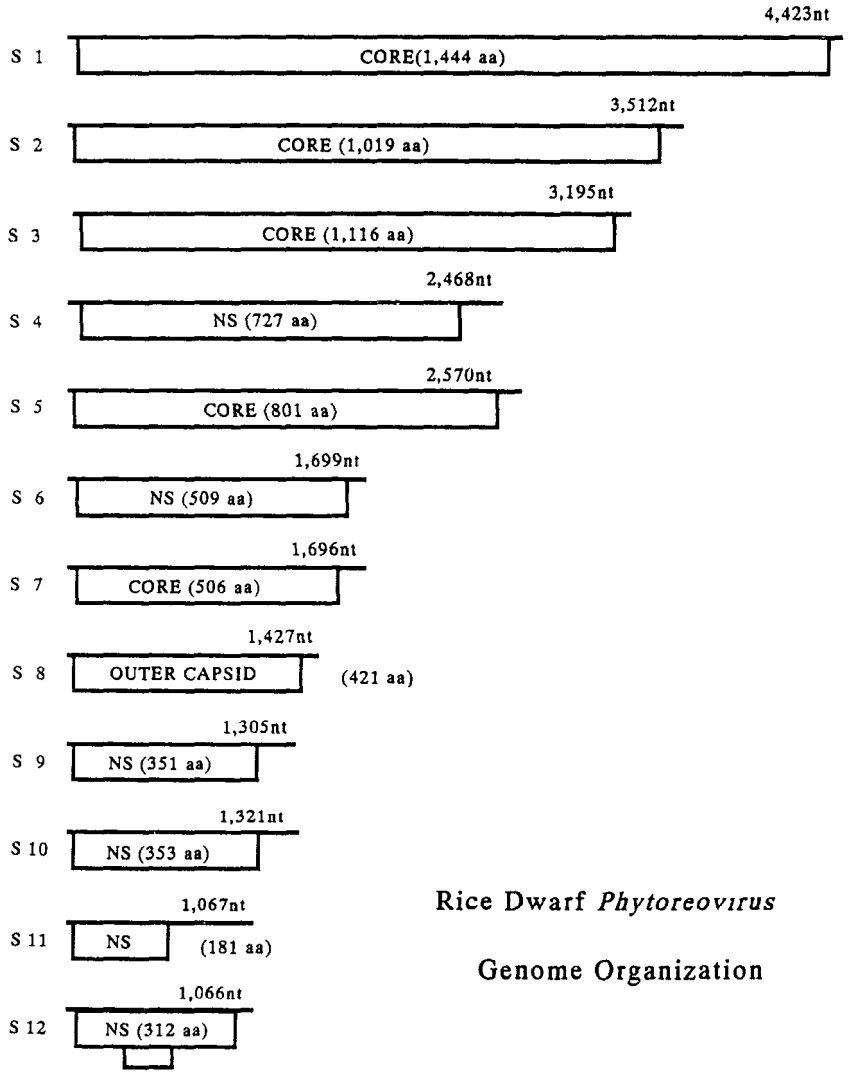



Fig 1 Genome organization of rice dwarf virus

low titer of the virus. A homogeneous virus culture can be obtained by serial transfers from one plant to another by an insect vector. Therefore, one subheading is devoted to virus isolation and propagation

The methods of genomic RNA extraction are described only for those from infected plants, not from purified virions. Since dsRNAs are rare components of a virus-free plant, genomic RNAs of plant reoviruses can be purified directly from infected plants relatively free from other nucleic acid components of plant origin; and they are pure enough to use for a polymerase chain reaction coupled

with reverse transcription (RT-PCR) and subsequent cDNA cloning into a bacterial plasmid vector

## 2. Materials

### 2.1. Isolation and Propagation of Rice Dwarf Virus

1. Insect culture. A colony of green leafhopper, *Nephotettix cincticeps,* is maintained on rice seedlings at 25°C under fluorescent lights for 16 h
2. 0.1*M* Phosphate buffer, pH 7.3 and pH 7.8, containing $KH_2PO_4$ and $Na_2HPO_4$
3. Requirements for microinjection into a vector insect are the following
   a. Stereoscopic microscope
   b. Fine glass needle. Stretch the 50-µL glass capillary (Drummond Scientific) with micropipet tension (Narishige, Model PB-7)
   c. 2-mL Glass syringe and silicone tubes to joint the needle and the syringe
   d. Petri dish (9-cm diameter)
   e. Compressed $CO_2$ gases to anesthetize insects
4. Rice seedlings for inoculation are prepared as follows. Soak 50–60 seeds in water for 2 d at 25°C to germinate. Transfer the individual seedling into a glass test tube (2.5-cm diameter and 13-cm length) containing 15–20 $cm^3$ of horticultural granular soil. Grow for 3–5 d

### 2.2. Purification of Viruses

#### 2.2.1. Rice Dwarf Virus

1. Virus source. Infected rice leaves and leaf sheaths showing clear symptoms 1–2 mo after inoculation
2. 0.1*M* Phosphate buffer, pH 6.0, containing $KH_2PO_4$ and $Na_2HPO_4$
3. Triton X-100
4. Chloroform
5. Carbon tetrachloride
6. 40% (w/v) Sucrose in 0.1*M* phosphate buffer, pH 6.0
7. Hitachi RP 30-2, RP 65, and RPS 27 rotors (or equivalents)
8. Glycerol

#### 2.2.2. Rice Black-Streaked Dwarf and Rice Ragged Stunt Viruses

1. Extraction (GMT) buffer. 0.3*M* glycine, 0.03*M* $MgCl_2$, 0.05*M* Tris-HCl, pH 7.5.
2. Carbon tetrachloride
3. Triton X-100
4. 40% (w/v) Sucrose in GMT buffer

### 2.3. Extraction of Viral RNAs and RT-PCR Amplification of Genomic dsRNAs

#### 2.3.1. Reagents for General Use

1. Milli-Q grade autoclaved $H_2O$.
2. TE. 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0

3. Phenol (nucleic acid grade): Equilibrate phenol with TE
4 Chloroform.isoamyl alcohol (24.1)
5 3*M* Sodium acetate, pH 5 2.

### 2.3.2. Direct Extraction of Genomic dsRNAs from Plants

1 10X STE buffer· 1*M* NaCl, 100 m*M* Tris-HCl, pH 6 8, 10 m*M* EDTA.
2 1X STE buffer Dilute the 10X STE buffer with sterile $H_2O$
3 CC41 cellulose powder (Whatman).

### 2.3.3. Direct Extraction of Viral mRNAs and Genomic dsRNAs from Plants

1 Milli-Q grade $H_2O$ treated with 0 1% DEPC and autoclaved
2 RNA extraction solution: 4*M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7 0, 0 5% sarcosyl, 0 1*M* 2-mercaptoethanol This solution is made by mixing the following
   a 250 g Guanidinium thiocyanate (Fluke) is dissolved in 293 mL Milli-Q grade $H_2O$
   b 17 6 mL 0 75*M* Sodium citrate, pH 7 0
   c 26 4 mL 10% Sarcosyl The stock solution (a + b + c) can be stored for at least 3 mo at room temperature
   d 0 36 mL 2-Mercaptoethanol/50 mL RNA extraction solution (a + b + c + d) can be stored for 1 mo at room temperature
3 Phenol (nucleic acid grade) Equilibrate phenol with Milli-Q grade $H_2O$
4 2*M* Sodium acetate, pH 4 0.
5 4*M* LiCl

### 2.3.4. RT-PCR of Genomic dsRNAs

1. Dimethyl sulfoxide (DMSO) (Spectrum grade). Flushed with $N_2$ gas
2 2.5 m*M* dNTPs mix, pH 8 0, in 1 m*M* Tris
3 Actinomycin D (500 µg/mL).
4 AMV reverse transcriptase XL (Life Sciences)
5 10X RTase buffer for AMV reverse transcriptase 500 m*M* Tris-HCl, pH 8 3 , 100 m*M* KCl, 40 m*M* DTT, 100 m*M* $MgCl_2$
6 Tth DNA polymerase and 10X reaction buffer supplied by the manufacturer
7 0 5 MEDTA, pH 8 0
8 Mineral oil (Sigma M-3516 or equivalent).

## 3. Methods

### 3.1. Isolation and Propagation of Rice Dwarf Virus

1 Preparation of inoculum Homogenize 10–50 mg of infected rice leaves in a small mortar and pestle with 19 times (v/w) of 0 1*M* phosphate buffer, pH 7 3. Transfer the homogenates to a 1.5 mL microtube, and centrifuge at 1800*g* for 5 min at 0°C Dilute the supernatant in 5–25 times of 0 1*M* phosphate buffer, pH 7 8, and use this extract as an inoculum

2 Collect the second instars of *N. cincticeps* in a small bottle and inject $CO_2$ gases into the bottle for 30–60 s to anesthetize. Arrange the insects on a Petri dish using a toothpick and cover them with Parafilm™ (American National Can) to immobilize.
3 Cut the point of the glass needle, and suck the inoculum into a glass needle with a syringe.
4 Inject a small amount of the inoculum (>1 μL) into the abdomen of each insect under a stereoscopic microscope.
5 Rear the insects on rice seedlings in a conical flask covered with a fine mesh screen for 10–12 d at 25°C under continuous fluorescent lights (latent periods).
6 Transfer individual insect to a rice seedling cv. Norin No. 8 grown for 5–7 d after germination in a test tube. Inoculate for 1–4 d by feeding.
7 Remove the insect, and grow the plant under fluorescent lights for 18 h at 25–27°C. About 1–2 wk after inoculation feeding, viral symptoms (white specks along veins) appear on new leaves.

## 3.2. Purification of Viruses

### 3.2.1. Rice Dwarf Virus

1 Harvest 10–50 g fresh rice leaves.
2 Homogenize the leaves with ELISA juice press (Erich Pollahne, Germany) in three to five times (v/w) of 0.1*M* phosphate buffer.
3 Add 1% (w/v) Driselase while stirring at 6°C with a magnetic stirrer for 1 h.
4 Add one-third vol of chloroform, mix the extract thoroughly for 3 min with Polytron homogenizer on ice.
5 Centrifuge at 3000*g* for 15 min.
6 Transfer the aqueous phase to a centrifuge tube, leaving interface. Centrifuge at 62,000*g* in a Hitachi RP 30-2 rotor for 60 min at 4°C.
7. Decant the supernatant. Add 4 mL of 0.1*M* phosphate buffer containing 1% Triton X-100, store the tube overnight at 4°C.
8 Dissolve the pellet thoroughly with Teflon homogenizer on ice. Add an equal volume of carbon tetrachloride; vortex for 3 min.
9 Centrifuge at 3000*g* for 15 min.
10 Transfer the aqueous phase to a centrifuge tube, leaving interface. Centrifuge at 80,000*g* in a Hitachi RP 65 rotor for 60 min at 4°C.
11 Decant the supernatant quickly, and dissolve the pellet in 1 mL of 0.1*M* phosphate buffer with Teflon homogenizer on ice.
12 Overlay the suspension on a 10–40% (w/v) linear sucrose density gradient, and centrifuge at 80,000*g* in a Hitachi RPS 27 rotor for 60 min at 4°C.
13 By inserting a L-shaped needle into the tube from the meniscus, collect the virus zone. Transfer into a centrifuge tube.
14. Centrifuge at 80,000*g* in a Hitachi RP 65 rotor for 60 min at 4°C.
15. Decant the supernatant quickly, and dissolve the pellet in a small amount of 0.1 *M* phosphate buffer.
16 Add an equal volume of glycerol, store at –80°C.

### 3.2.2. Rice Black-Streaked Dwarf and Rice Ragged Stunt Viruses

1 Fresh infected leaf and sheath tissue (100 g) (rice for RRSV and corn for RBSDV) are cut into about 1-cm pieces and ground with a meat grinder with 35 mL GMT buffer
2 The homogenate is further extracted with a ELISA juice press
3 Squeeze through a double layer of medical gauze.
4 Add 30% (v/v) carbon tetrachloride and 3% (v/v) Triton X-100 while stirring with a magnetic stirrer at 4°C for 1 h
5 Centrifuge at 1500*g* for 20 min in an angle rotor at 4°C
6. The aqueous phase is centrifuged at 80,000*g* for 1 5 h at 4°C through one-third vol of a tube capacity of 40% sucrose in GMT buffer in a RPS 27 rotor
7 Suspend the pellet in 2 mL of GMT buffer.
8 Centrifuge in a microcentrifuge tube at 3500*g* for 5 min at 4°C
9 The supernatant is layered onto a 10–40% sucrose density gradient tube and centrifuged at 80,000*g* for 1 5 h at 4°C in a RPS 27 rotor
10. Recover the virus zone at the middle of the tube, using an ISCO density gradient fractionator equipped with an UV monitor

## 3.3. Direct Extraction of Viral RNAs from Plants

### 3.3 1. Direct Extraction of Viral Genomic dsRNAs from Plants

1 Extract the virus with ELISA juice press from 0 05–0 50 g of infected rice leaves, while adding 600 µL of STE buffer into a microcentrifuge tube
2 Add an equal volume of phenol.
3 Vortex for 3 min
4 Centrifuge at 10,000*g* for 1 min at room temperature
5. Transfer the aqueous phase (normally, the aqueous forms the upper phase) to a fresh microcentrifuge tube
6 Add an equal volume of phenol chloroform·isoamyl alcohol (25 24 1) into the aqueous phase and repeat **steps 3–5**
7 Add 80 mg of CC41 cellulose (Whatman) powder and 0 2 vol of ethanol into the microcentrifuge tube containing the aqueous phase Agitate the mixture for 30 min at room temperature
8 Collect the cellulose by centrifugation at 10,000*g* for 3 min
9 Add 1 2 mL STE buffer, pH 6 8, containing 15% ethanol, into the pellet of cellulose after removing the supernatant Vortex for 1 min
10 Repeat the washing of **steps 8** and **9** once more.
11. Elute dsRNA by adding 150 µL of sterile $H_2O$ and vortex for 1 min
12 After centrifugation at 10,000*g* for 3 min, transfer aqueous phase to a fresh microcentrifuge tube Repeat the elusion of **steps 11** and **12** once more Combine the second aqueous phase with the first
13 Remove traces of cellulose by centrifuging briefly the combined aqueous phase and transfer the supernatant into a fresh microcentrifuge tube

14 Add 0 1 vol of 3.0*M* sodium acetate, pH 5.2, and 2 5 vol of ethanol, mix, and then incubate for 60 min on ice
15 Precipitate dsRNA by centrifugation at 15,000*g* for 15 min at 4°C in the microcentrifuge
16 Remove supernatant and then wash the pellet with 1 mL of 70% ethanol
17. Recover the pellet by centrifugation at 15,000*g* for 10 min at 4°C in the microcentrifuge
18 Stand the open tube on the bench at room temperature until the last traces of fluid have evaporated
19 Dissolve the dsRNA pellet (which is often invisible) in the desired volume of TE or sterile $H_2O$. Rinse the walls of the tube well with the buffer or sterile $H_2O$

### *3.3.2. Direct Extraction of Viral mRNAs and Genomic dsRNAs from Plants*

1 Infected fresh rice leaves are homogenized with the RNA extraction solution (500 µL/100 mg) in a mortar and pestle
2 Transfer the homogenate to a 1 5-mL microcentrifuge tube and centrifuge for 1 min at high speed
3 Transfer 500 µL supernatant to a new microcentrifuge tube
4 Add 50 µL 2*M* sodium acetate and 500 µL phenol, 100 µL chloroform isoamyl alcohol Mix thoroughly by inverting the tube after the addition of each reagent
5 Vortex for 10 s
6 Stand the tube for 3 min at room temperature
7 Centrifuge for 15 min at 10,000*g* at 4°C
8 Transfer the aqueous phase to a new microcentrifuge tube
9. Add 500 µL isopropanol
10 Stand for 5–10 min at room temperature
11 Centrifuge for 10 min at 10,000*g* at 4°C
12 The RNA pellet is washed with 1 mL 70% ethanol
13 Dry the pellet for 5 min under vacuums
14 Suspend in 250 µL sterile $H_2O$
15 Add 250 µL 4*M* LiCl and vortex.
16 Place on ice for more than 8 h
17 Centrifuge for 10 min at 10,000*g* at 4°C
18 Supernatant contains dsRNAs and tRNAs Collect them by ethanol precipitation dsRNAs are further purified by CC41 treatment as in **Subheading 3.3.1., step 7** The pellet contains viral mRNAs suitable for Northern blotting analyses

## *3.4. RT-PCR Amplification of Genomic dsRNAs*

### *3.4 1. Basic RT-PCR*

1 Add 100 µL of DMSO to 5 µg of genomic dsRNAs in 5 µL sterile $H_2O$
2 Incubate at 50°C for 30 min
3 Add 10 µL 3*M* sodium acetate and 300 µL ice-cold ethanol

4. Incubate at –80°C for 30 min or place in dry ice for 5 min
5. Centrifuge at 16,000*g* for 10 min at 4° C
6. Remove supernatant, rinse the precipitate with 1 mL 70% ethanol
7. Dry under vacuum
8. Make up the following master mix for one sample
   a. 5 μL 10X RTase buffer
   b. 5 μL Actinomycin D
   c. 20 μL 2 5 m*M* dNTP mix
   d. 1 μL 3' Antisense-strand primer (100 pmol/1 μL)
   e. 19 μL Sterile $H_2O$
   f. 0 3 μL AMV reverse transcriptase XIL (30 U/μL)
9. Add the master mix to the dried denatured genomic RNAs, vortex, and spin briefly
10. Incubate at 45°C for 1 h and terminate the reaction by adding 1 μL 0 5*M* EDTA, pH 8.0.
11. Add 25 μL each of phenol and chloroform isoamyl alcohol (24 1)
12. Vortex for 3 min and centrifuge for 5 min
13. Transfer the aqueous phase to a new microcentrifuge tube and add 50 μL chloroform
14. Vortex for 3 min and centrifuge for 5 min.
15. Transfer the aqueous phase to a new microcentrifuge tube and add 0 1 vol of 3*M* sodium acetate and 2 5 vol of ethanol
16. Place on ice for 30 min and centrifuge at 16,000*g* for 10 min at 4°C
17. Rinse the precipitate with 70% ethanol and dry under vacuums
18. Suspend in 25 μL sterile $H_2O$ and 5 μL was subjected to a PCR reaction
19. In a 0 5-mL microcentrifuge tube, mix in the following in order
    a. 5 μL 10X amplification buffer.
    b. 8 μL 1 25 m*M* dNTPs
    c. 1 μL 5' Sense-strand primer (100 pmol/1 μL).
    d. 1 μL 3' Antisense-strand primer (100 pmol/1 μL)
    e. 30 μL Sterile $H_2O$.
    f. 1 μL Tth DNA polymerase (4U/μL)
    g. 5 μL First-strand cDNA made from up to 1 μg dsRNA.
20. Overlay the reaction mixture with one drop (about 30 μL) of light mineral oil and briefly spin
21. Carry out the amplification of cDNAs with a thermal cycler Typical conditions for the synthesis of cDNA, denaturation, annealing, and polymerization are as follows

| | |
|---|---|
| 24 PCR cycles | 94°C for 1 min |
| | 55°C for 2 min |
| | 72°C for 3 min |
| Final extension | 72°C for 10 min |

22. Withdraw a portion of the amplified DNAs from the reaction mixture and analyze it by gel electrophoresis, Southern hybridization, or DNA sequencing

### *3.4.2. Single-Step RT-PCR*

1 In a 0.5-mL microcentrifuge tube, mix in the following order
   a 5 μL 10X amplification buffer
   b. 5-μL Mixture of four dNTPs, each of 5 m*M*
   c 1 μL 5' Sense-strand primer (100 pmol)
   d 1 μL 3' Antisense-strand primer (100 pmol)
   e 30 μL Sterile $H_2O$
   f 1 μL Tth DNA polymerase (4U/μL)
   g 0 3 μL AMV reverse transcriptase XL (30 U/μL)
   h 5 μL Template dsRNA denatured with DMSO (up to 1 μg)
2 Overlay the reaction mixture with one drop (ca 30 μL) of light mineral oil and briefly spin
3. Carry out the cDNA synthesis and amplification of cDNAs in the same tube with a thermal cycler Typical conditions for the synthesis of cDNA, denaturation, annealing, and polymerization are as follows

| | |
|---|---|
| One cycle for cDNA synthesis | 42°C for 15 min |
| An initial denaturation | 95°C for 1 min |
| 30 PCR cycles | 95°C for 1 min |
| | 55°C for 2 min |
| | 72°C for 3 min |
| Final extension | 72°C for 10 min |

4 Withdraw a sample of the amplified DNA from the reaction mixture and analyze it by gel electrophoresis, Southern hybridization, or DNA sequencing

## 4. Notes

1 Equipment for handling leafhoppers and planthoppers, such as cages for maintaining and tools for transferring the insects, are described elsewhere ***(6)***. Injection of viruses into the vector insects is a good alternative to a conventional acquisition feeding Nearly 100% of the injected insects become viruliferous The younger the larvae, the more they recover and survive after injection of the viral extract Use larvae or male adults for inoculation feeding to prevent laying eggs into inoculated plants Hatching eggs on the inoculated plants becomes a serious source for contamination of viral cultures Most rice cultivars are not resistant to virus infection, but some are resistant to vector insects The cultivar Norin no 8 is used in our laboratory and TN 1 is used worldwide (**Subheading 3.1.**)
2 Since individual virus isolates have similar but distinct electrophoretic mobility of the genomic segments ***(5)***, viral cultures collected from fields should be examined for genomic homogeneity by PAGE of genomic dsRNAs, 50–100 ng of dsRNAs should give clear bands in 40-cm-long and 0 8-mm-thick gel after silver staining (**Subheading 3.1.**)
3 Plant materials for purification of RBSDV and RRSV are very critical for obtaining a good yield Young and fresh leaves showing good symptoms within 1 mo

after inoculation should be used Freezing the plant materials drastically reduces the virus yield In the case of RRSV, no virus can be recovered after freezing of infected rice plants

The purification protocol described here *(7)* was originally developed for maize rough dwarf virus and works well for both RBSDV and RRSV Rice ragged stunt virus can be purified by other methods as well *(8,9)* (**Subheading 3.2.2.**)

4 At the second organic solvent treatment during the purification of viral particles, we have previously used freon Since freon is not available anymore, carbon tetrachloride is used as a substitute (**Subheading 3.2.1., step 8**)

5 Techniques involved in extraction of dsRNAs are reviewed in detail by Dodds et al *(15)* Direct extraction of genomic dsRNAs described here is a modification of a method described by Dodds et al *(10)* The major modification is a use of CC41 *(11)*, instead of CF11 cellulose Fine granular texture of CC41 makes it easier to handle by a batch method in a microcentrifuge tube An additional modification is that the STE buffer does not contain mercapthoethanol, SDS, and bentonite at an extraction step Quality of dsRNAs obtained from RDV-infectecl rice plants is pure enough to subject to RT-PCR So far as we tested with RDV S8, S9, and S10, we have successfully amplified full-length cDNAs We have not yet tested this protocol for RRSV and RBSDV (**Subheading 3.3.1.**)

Our standard procedure for direct extraction of the genomic dsRNAs includes additional steps After the phenol and chloroform extraction, total nucleic acids are precipitated by ethanol and suspended in 100–400 µL of TE, and then an equal volume of 4*M* LiCl is added to precipitate high-mol-wt ssRNAs *(12)* Supernatants containing the dsRNAs are then treated with CC41, exactly as described by Dlieu and Bar-Joseph *(11)*. The standard method has been used routinely in our laboratory for purifying genomic dsRNAs of RDV *(5)*, RBSDV, and RRSV *(13)*. The method should yield ~5–10 µg from 0 5 g of infected leaves for RDV, 0 1 µg for RBSDV, and 0 5 µg for RRSV Using genomic dsRNA templates prepared by this method, we have successfully amplified full cDNAs of RDV S4, S5, S6, S7, S8, S9, S10, S11, and S1 2 by the basic protocol of RT-PCR described in **Subheading 3.4.1.**

A method for extraction of both viral mRNAs and genomic dsRNAs described here is based on a protocol of Chomczynski and Sacchi *(14)* (**Subheading 3.3.2.**)

## References

1 Uyeda, I and Milne, R G (1995) Introduction genome organization, diversity and evolution of plant reoviruses *Semin Virol* **6,** 85–88

2 Uyeda, I , Suda, N , Yamada, N , Kudo H , Murao, K , Suga, H , Kimura, I , Shikata, E , Kitagawa, Y , Kusano, T , Sugawara, M , and Suzuki, N (1994) Nucleotide seqeunce of rice dwarf Phytoreovirus genome segment 2 completion of sequence analyses of rice dwarf virus *Intervirology* **37,** 6–11.

3 Suzuki, N (1995) Molecular analysis of the rice dwarf virus genome *Semin Virol* **6,** 89–95

4 Uyeda, I , Kimura I , and Shikata, E. (1995) Characterization of genome structure and establishment of vector cell lines for plant reoviruses *Adv Virus Res* **45,** 249–279

5 Murao, K , Suda, N , Uyeda, I , Isogai, M , Suga, H , Yamada, N , Kimura, I , and Shikata, E (1994) Genomic heterogeneity of rice dwarf phytoreovirus field isolates and nucleotide sequences of genome segment 12 *J Gen Virol* **75,** 1843–1848

6. Heinrichs, E A , Medrano, F G , and Rapusas, H R (1985) *Genetic Evaluation for Insect Resistance in Rice,* International Rice Research Institute, Los Banos, Philippines

7 Redolfi, P and Boccardo, G. (1974) Fractionation of the doublestranded RNA of maize rough dwarf virus subviral particles *Virology* **59,** 319–322

8 Kawano, S , Shikata E , and Senboku, T (1983) Purification and morphology of rice ragged stunt virus *J Fac Agr Hokkaido Univ* **61,** 209–218

9. Omura, T , Minobe, Y , Kimura, I., Hibino, H., Tsuchizaki, T , and Saito, Y. (1983) Improved purification procedure and RNA segments of rice ragged stunt virus *Ann Phytopathol Soc Japan* **49,** 670–675

10 Morris, T J , Dodds, J A , Hillman, B , Jordan, R L , Lommel, S A , and Tamaki, S J (1983) Viral specific dsRNA· diagnostic value for plant virus disease identification *Plant Mol Biol Rep* **1,** 27–30.

11 Dlieu, P and Bar-Joseph, M (1989) Rapid isolation of double stranded RNA segments from disulphide crosslinked polyacrylamide gels *J Virol Methods* **24,** 77–84

12 .Diaz-Ruiz, J R and Kaper, J N (1978) Isolation of viral doublestranded RNAs using LiCl fractionation procedure *Prep Biochem* **8,** 117

13 Suga, H., Uyeda, I., Yan, J , Murao, K , Kimura, I , Tiongco, E R , Cabauatan, P , and Kcganezawa, H (1995) Heterogeneity of rice ragged stunt oryzavirus genome segment 9 and its segregation by insect vector transmission *Arch Virol* **140,** 1503–1509

14 Chomczynski, P and Sacchi, N (1987) Single-step method of RNA isolation by acid guanidinium lthiocyanate-phenol-chloroform extraction *Anal Biochem* **162,** 156–159

15 Dodds, J A , Morris, T J , and Jordan, R L (1984) Plant viral doublestranded RNA *Ann Rev Phytopathol* **22,** 151–168

# 7

# Procedures for Plant Rhabdovirus Purification, Polyribosome Isolation, and Replicase Extraction

**Andrew O. Jackson and John D. O. Wagner**

## 1. Introduction

The *Rhabdoviridae* family consists of a large number of nonsegmented negative-strand RNA viruses. As a group, the rhabdoviruses cause many serious plant and animal diseases that have detrimental effects on agricultural productivity, public health, and wildlife populations. The members of the family collectively have an unusually broad host range composed of viruses that infect both plants and animals *(1)* Many of these viruses are persistently transmitted to their mammalian and plant hosts by insect or arthropod vectors in which they are able to multiply Consequently, these members of the family may have been able to expand their evolutionary diversity by use of their vectors as intermediate hosts to bridge the boundaries between the animal and plant taxa. Other rhabdoviruses are known to infect fish and aquatic invertebrates, and probably are transmitted via contaminated water.

Rhabdovirus particles are recognized easily in plants by electron microscopic observation of sap from diseased tissue or in thin sections of infected cells *(2)*. The virions are normally bacilliform if extracts are fixed in glutaraldehyde prior to negative staining, but are bullet shaped if the fixative is omitted Because the particles, with sizes reported to range from 45 to 100 nm wide and 150 to 400 nm long, can be distinguished so readily from the constituents present in uninfected tissue, numerous possible rhabdovirus diseases have been described in many different plant families *(2)*. Microscopy of thin sections of infected cells reveals that the particles of different members normally have two characteristic patterns of accumulation: they are found either in association with the nucleus or in the cytoplasm. The *Sixth Report of the International Committee on Taxonomy of Viruses (3)* has used these subcellular distribution

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

patterns to separate plant rhabdoviruses into two major taxonomic groups. the *Cytorhabdovirus* genus and the *Nucleorhabdovirus* genus.

Presently, eight viruses (barley yellow striate mosaic virus, broccoli necrotic yellows virus, Festuca leaf streak virus, lettuce necrotic yellows virus, northern cereal mosaic virus, Sonchus virus, strawberry crinkle virus, and wheat American striate mosaic virus) are assigned to the *Cytorhabdovirus* genus. The *Nucleorhabdovirus* genus has six members (Datura yellow vein virus, eggplant mottled dwarf virus, maize mosaic virus, potato yellow dwarf virus, Sonchus yellow net virus, and sowthistle yellow vein virus). Of these, Sonchus yellow net virus (SYNV) and lettuce necrotic yellows virus (LNYV) have been the most extensively characterized. Less extensive cytopathological and physicochemical information is available about other rhabdoviruses within the *Cytorhabdovirus* and *Nucleorhabdovirus* genera. Only preliminary and less reliable descriptions are available for other plant rhabdoviruses, consequently, more than 60 members and possible members have yet to be assigned to a genus *(3)*.

The bacilliform or bullet-shaped rhabdovirus virions are complex, with three distinct layers varying in electron density observed in high resolution electron micrographs (**Fig. 1A**). These layers appear to represent glycoprotein surface projections, an outer membrane, and a helical striated inner nuclear core with a central canal (**Fig. 1B**). The membrane contains host-derived lipids and glycoprotein spikes that probably associate as trimers and protrude 5–10 nm through the membrane. The nucleocapsid contains three proteins, designated N (nucleocapsid), P (phosphoprotein), and L (polymerase), that encapsidate the negative-strand genomic RNA This RNA ranges in size from 11 to 14 kb, depending on the virus. A matrix (M) protein is thought to mediate coiling of the nucleocapsid and its association with the membrane. Analogs of these five proteins have been found in all rhabdoviruses that have been carefully analyzed. A sixth protein is encoded by the genomes of some rhabdoviruses. In SYNV, this gene, which we have provisionally designated sc4, is virion-associated and has no obvious sequence relatedness to the sixth accessory proteins of other rhabdoviruses *(4)*. The entire genome of SYNV has been sequenced; consequently, we have available a detailed genetic map (**Fig. 1C**), and have considerable information about the nature of the viral proteins (*see* **ref. *4*** and references therein) A genome map *(5)* and limited sequence analysis of LNYV is also available *(6)*.

The methods that have the broadest spectrum of applicability for purification of rhabdoviruses have arisen from studies of SYNV and LNYV. We have described the method developed for SYNV in this review because it has been successfully used for a large number of rhabdoviruses. An earlier procedure was developed for LNYV, which used chromatography over calcium phosphate gels, and the last iteration of the protocol has been described in some

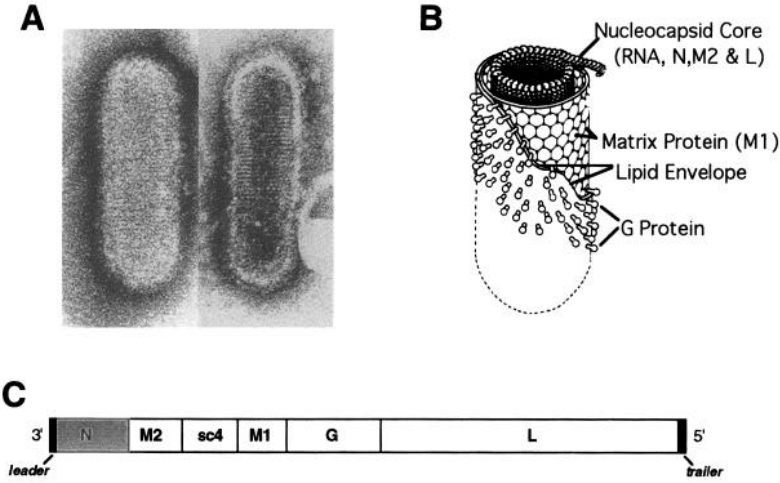



Fig. 1. Rhabdovirus particle morphology and Sonchus yellow net virus genome map. **(A)** Electron micrograph of negatively stained particles of SYNV. The negative stain reveals the surface structure of the particle on the left, and deeper penetration reveals the core of the particle on the right. Note the typical internal striated nucleocapsid core and the projecting spikes surrounding the membrane. **(B)** Model illustrating the components of rhabdovirus particles. The helical nucleocapsid core consists of the genomic RNA, the nucleocapsid (N) protein, the phosphoprotein (M2), and the L protein. A matrix protein (M1) is involved in attachment of the envelope to the nucleocapsid. This membrane consists of host lipids interspersed with an orderly array of glycoprotein (G) spikes. A sixth protein, sc4, is associated with the membrane, but its location or contribution to the particle morphology is not known. **(C)** Drawing illustrating the organization of the genes encoded by the 13,760-nucleotide genome of Sonchus yellow net virus. The genome order from the 3' to the 5' end of the (–)-strand RNA is presented from the left to right, according to convention. The genes consist of the leader sequence, the nucleocapsid (N) gene, the phosphoprotein (M2) gene, a gene encoding an envelope protein (sc4) of unknown function, the glycoprotein (G) gene, the polymerase protein (L) gene, and the trailer sequence. The relative size of each gene is proportional to the size of the viral RNA. A and B were adapted from **refs. *2*** and ***10***, respectively.

detail by Francki et al. ***(7)***. The concepts developed by the late Richard Francki, and his encouraging advice were also of enormous assistance during the development of the SYNV procedure ***(8)***. Studies of the replication of SYNV have also required development of several other techniques that may be applied directly to studies of virus replication, so we have included two additional procedures that we believe may have general applicability to studies of plant rhabdoviruses. These include techniques developed for isolation of polyribosomes

*(9)* and a polymerase complex from nuclei of plants infected with SYNV *(10)* These techniques each permit analysis of transcription and translation of SYNV genes in vivo.

### 1.1. Problems Encountered During Purification of Plant Rhabdoviruses

The single most important factor limiting studies of plant rhabdoviruses is the difficulty of devising simple and reproducible purification protocols suitable for recovery of adequate amounts of highly purified virus for biochemical analyses. Therefore, we have provided a synopsis emphasizing the factors that have proven to be important for optimizing recovery of highly purified virus that retains its infectivity.

Before embarking on development of a purification procedure, several factors related to the interaction of the virus with the host should be considered in order to obtain good virus yields. If the virus is virulent on several hosts, the particular hosts or cultivars that give the highest infectivity titers should be investigated further. Identification of suitable hosts for purification was particularly important for development of purification protocols for SYNV, potato yellow dwarf (PYDV), and LNYV. In some cases, the choice of the host was critical. For instance, strawberry crinkle virus (SCV) was successfully purified *(11)* only after it was transferred from strawberry into *Physilis floridana*. Since SCV could be mechanically transmitted from *Physilis,* this eliminated the necessity of using the aphid vector for routine serial transfers, and it also permitted mechanical transmission to a range of experimental hosts that are not preferred by the vector. Even so, we were unable to purify the virus from *Nicotiana edwardsonii* or *N. glutinosa,* both of which had strikingly intense symptoms. In addition to the host used for purification, the age of the plants, and the light and temperature requirements, as well as the length of infection, are critical factors that can drastically alter the amount of virus recovered from tissue. As described below, these factors are extremely important for purification of optimal amounts of SYNV. Thus, to obtain the highest yields and purity of rhabdoviruses, one must consider a number of host and environmental variables. However, the time invested in a systematic analysis of host and biological factors that contribute to optimum recovery of rhabdoviruses can help minimize numerous problems that otherwise might arise during subsequent development of purification protocols *(2)*.

A reliable procedure for virus detection during different stages of purification is a second important consideration that can help in determining the efficacy of procedures used for virus separation, and minimize the effort necessary to optimize a purification protocol. Electron microscopy is an obvious choice for following particle enrichment, but the labor, precision, and expense of this

technique compromises its utility, and no indication of biological activity can be obtained by microscopy observations alone. Therefore, when available, an infectivity assay should be used in conjunction with physical methods of detection. In the case of SYNV, LNYV, and PYDV, local lesion hosts have provided a suitable assay to monitor the amount of virus in different subcellular fractions. In a few specific cases, tissue cultures or cultured explants from insects that are vectors of the virus have been used for bioassays *(12)*. Even though these cultures are difficult to maintain and have not been established from vectors of most rhabdoviruses, they do provide the most sensitive and rapid bioassays yet devized for plant viruses. Chemical methods may also provide a useful means of detection, especially when combined with electron microscopy and biological assays For example, glycoprotein detection using specific lectins, in combination with Coomassie blue staining of polyacrylamide gels, has been used to assess the effectiveness of the early stages in purification *(11,13)*. Use of an antiserum, and even antibody preparations that have some reactivity to host components when used in Western blots, can also be of enormous help for evaluating the presence and concentration of virus particles at different stages of purification.

In general, yields of rhabdoviruses from infected plants are low compared with other plant viruses, and the membrane-containing particles are often difficult to separate from host components. These problems are often compounded because of the lability of the virions The ease with which rhabdovirus virions lose infectivity prohibits commonly used heating and freezing treatments, or the pH adjustments frequently used for clarification. Moreover, the common organic solvents and the mild detergents used to purify simple RNA viruses will solubilize the lipid membranes of rhabdovirus particles However, several general components of the extraction media that expedite purification of plant rhabdoviruses, such as the appropriate pH, a high osmoticum, and the presence of reducing agents and divalent cations, have been known for more than 20 yr *(14)* More specific requirements for stability of several individual rhabdoviruses have been determined more recently, and these should provide valuable guidelines for developing purification protocols for uncharacterized rhabdoviruses. The reader is referred to Jackson et al. *(2)* for a more elaborate description of the requirements needed for these viruses than can be accommodated here.

Almost all purification schemes devised for the rhabdoviruses have relied on some form of centrifugation to concentrate the virus. Unfortunately, rhabdovirus preparations obtained by centrifugation without prior clarification are normally too contaminated with host components for even crude chemical characterization The most difficult contaminants to remove are chloroplast and membrane fragments, and the presence of these components appears to be a major cause of irreversible virus aggregation following pelleting by high-speed

centrifugation. Therefore, host components must be selectively removed without substantial virus loss before concentration of the virions. Selective filtration normally provides the most effective method to separate rhabdoviruses from plant host contaminants. For this purpose, rhabdovirus preparations are often filtered through thin pads of Celite before concentration *(2)*. When Celite pads of appropriate thickness are used, and the washing steps are optimized, greatly enriched virus particles pass through the Celite pads before significant elution of host chloroplast and mitochondrial membrane fragments occurs However, filtration through other supports may be useful with certain rhabdoviruses For example, LNYV has been clarified by shaking extracts with DEAE cellulose and decolorizing charcoal or bentonite before Celite filtration *(14)*. Unfortunately, these clarification procedures are not equally effective with all rhabdoviruses, because DEAE cellulose, bentonite, and various clays can adsorb other rhabdoviruses (A. O. Jackson, unpublished observations).

After clarification, rhabdoviruses are usually concentrated from homogenized plant brei by differential centrifugation. Unfortunately, because of particle lability, the reduction in biological activity caused by compression forces during ultracentrifugation is more pronounced with rhabdoviruses than with simpler RNA viruses. Even with SYNV, with which we have routinely used differential centrifugation, the infectivity of the virus is reduced when clarified extracts are centrifuged at high speed *(8)*. Therefore, we have also employed a simple polyethylene glycol (PEG) precipitation procedure as an alternative to differential centrifugation to prepare highly infectious SYNV *(15)*. Thus, when it is important to maintain infectivity, precipitation with PEG, followed by low-speed centrifugation, provides a suitable alternative to ultracentrifugation. However, PEG concentrations required for optimum precipitation of rhabdoviruses vary somewhat *(12)*, and this will need to be evaluated with individual viruses.

Following concentration, rhabdoviruses are normally subjected to rate-zonal and equilibrium centrifugation in sucrose gradients during the final stages of purification. However, various types of chromatography have been used in a few limited cases in the final stages of purification *(2)* Chromatography over calcium phosphate is routinely used as a step in purification of LNYV *(7)* However, this method is somewhat limited in utility, since different batches of calcium phosphate may not be uniform (R. I. B. Francki, personal communication), and our experiments have also shown that there is considerable variation in the absorption of SYNV and PYDV by calcium phosphate (A O Jackson, unpublished observations) Electrophoresis into sucrose gradients has also been used, but major disadvantages with this method are the time involved and possible losses in infectivity. Therefore, although these alternative steps may have some utility for specific purposes, neither method is really satisfactory for general use with rhabdoviruses.

## 2. Purification of Sonchus Yellow Net Virus

### 2.1. Introduction

The procedure used for SYNV has been adapted for use with several other rhabdoviruses. This method was initially developed by Jackson and Christie *(8)* in conjunction with an infectivity assay using the local lesion host *Chenopodium quinoa* (*see* **Subheading 2.4., Note 1**). The local lesions formed after inoculation permitted us to evaluate the most appropriate host, the optimum greenhouse conditions for virus replication, and the relative levels of infectivity at different times after inoculation of plant tissue, as a prelude to development of the purification protocol. These bioassays revealed that *N edwardsonii* is a suitable host for virus maintenance and recovery, and subsequent experiments have shown that equal or better yields can be recovered from *N. benthamiana*. Local lesion assays were also valuable for assessing virus recovery after each purification step. As the procedure developed, particular attention was directed to reproducible removal of host components.

### 2.2. Materials

1. Inoculation buffer: Shortly before use, prepare 40 m*M* sodium sulfite ($Na_2SO_3$), containing Celite as a mild abrasive by adding 125 mg of $Na_2SO_3$ and 500 mg (2%) of Celite Analytical Filter Aid (Johns-Mansville, Denver, CO) to 25 mL of $H_2O$. Store on ice and mix well immediately before use.
2. Extraction buffer. Add 60 g of Tris base, 1.1 g of Mg acetate, and 120 mg of $MnCl_2$ to 450 mL of $H_2O$. Adjust the pH to 8.4 with HCl. Store at 4°C until just before use, then add 2.5 g of $Na_2SO_3$ and bring the volume to 500 mL. The final extraction buffer is 100 m*M* Tris-HCl, pH 8.4, 10 m*M* Mg acetate, 1 m*M* $MnCl_2$, and 40 m*M* $Na_2SO_3$.
3. Maintenance buffer: Maintenance buffer is identical to extraction buffer, except that the pH is adjusted to 7.5.

### 2.3. SYNV Purification Method

1. Transfer SYNV at 14-d intervals by inoculating *N. benthamiana* or *N edwardsonii* plants grown in 15-cm clay pots containing autoclaved loam soil. Prepare inoculum by macerating 2 g of infected leaves in a chilled mortar containing 5 mL of freshly prepared cold (~4°C) inoculation buffer (*see* **Subheading 2.4., Note 2**). Gently rub the leaves with cheesecloth dipped into the leaf extract. Under optimum growth conditions in the greenhouse (~25°C) and normal summer sunlight, the light yellow netting symptoms characteristic of SYNV begin to appear on the leaves by 8–10 d after inoculation.
2. Harvest systemically infected leaves, with the midribs and petioles, including the youngest rosette leaves, where the most intense symptoms are normally found. The inoculated leaves generally contain much lower titers and are not harvested. Either extract immediately or store for several days at 4°C.

3 Blend 60 g of leaves for 1 min in 120 mL of cold (~4°C) extraction buffer Squeeze the brei through two layers of cheesecloth and centrifuge immediately at 4000*g* for 10 min in a low-speed fixed-angle rotor (in a Sorval GSA rotor)
4 While the centrifuge is running, make six discontinuous gradients in 25 × 89-mm centrifuge tubes for a Beckman SW28 rotor. Pipet 8 mL of 600 mg of sucrose/mL in maintenance buffer into each tube to form the bottom layer Then, gently pipet 5 mL of 300 mg/mL sucrose over the bottom layer to make a distinct interface between the two layers (*see* **Subheading 2.4., Note 3**)
5 Adjust the supernatant recovered from the low-speed centrifugation to pH 7 5 Then, load 20-mL aliquots on the top of each of the six discontinuous gradients Centrifuge the gradients for 60 min at 4°C at 110,000*g* in a Beckman SW 28 rotor to concentrate the virus between the 300 and 600 mg/mL sucrose layers (*see* **Subheading 2.4., Note 4**)
6 Prepare a Celite filter pad during the 60-min centrifuge run For this purpose, suspend 17 g of Celite in about 100 mL of maintenance buffer. Pour the slurry onto a Whatman 3MM filter paper in a 10-cm Buchner funnel Insert the funnel into a 1-L sidearm flask attached to a strong vacuum pump Pull a vacuum and quickly release the vacuum just before the liquid reaches the top of the pad. Then, gently pour 100 mL of maintenance buffer over the pad and suck this through the pad under a vacuum Again, release the vacuum just as the liquid reaches the top of the pad The pad should be firm, with just a slightly wet sheen Gently pipet about 5 mL of maintenance buffer over the pad and store upright, attached to the sidearm flask (*see* **Subheading 2.4., Note 5**).
7. Collect the green band between the 300 and 600 mg/mL sucrose layers from the tubes with a 15-gage or larger diameter bore needle, bent at a right angle near the tip, and attached to a 50-mL syringe Dilute the green material (usually about 30 mL total) with an equal volume of maintenance buffer that had been allowed to equilibrate to 4°C Stir 1 g of Celite into the suspension
8 Gently suck the maintenance buffer through the Celite pad with a vacuum Again, leave a slight sheen at the top of the pad Swirl the plant material recovered from the sucrose interface and gently layer it over the Celite pad while pulling a stronger vacuum Try not to disturb the surface of the pad. When the green slurry is about 5 mm above the pad, slowly begin to add maintenance buffer to the pad, and wash with 100 mL of the buffer. The filtrate should be a light-tan color and should exhibit light scattering when held up to a focused light source (*see* **Subheading 2.4., Note 6**)
9 Pour the filtrate into 30-mL tubes and centrifuge at 90,000*g* in a fixed-angle rotor in a Beckman Type 30 rotor for 30 min at 4°C to pellet the virus Small light-tan to slightly green pellets about 5 mm in diameter will usually be visible The color of these pellets is an excellent indicator of the final purity that can be expected Quickly aspirate the solution from the pellets and resuspend them in a total volume of 1 mL of maintenance buffer
10. Layer the suspension over a rate-zonal sucrose gradient formed 12–24 h previously by layering 5-, 10-, 10-, and 10-mL layers, respectively, of 50, 100, 200, and 300 mg of sucrose/mL of maintenance buffer

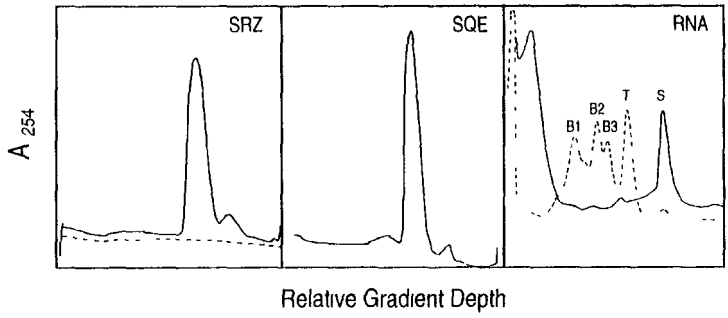



Fig 2 Sedimentation of purified SYNV and viral RNA SRZ panel. Patterns from SYNV-infected tobacco (solid line) and uninfected tobacco (dashed line) on rate-zonal sucrose gradients The preparations were separated from tobacco and centrifuged in the gradients after clarification by Celite filtration and concentration by high-speed centrifugation The particles have a sedimentation coefficient estimated at 1044 S SQE panel Banding of the particle recovered from SRZ in quasiequilibration sucrose gradients. The particles have a density of 1 18 g/mL in sucrose RNA panel Comparison of the sedimentation rates of SYNV RNA (S) with the three RNAs of brome mosaic virus (B-1, B-2, and B-3), and tobacco mosaic virus RNA (T) Note that the purity of the preparation can be determined from the amounts of the ribosomal RNAs sedimenting near BMV RNAs 2 and 3 Modified from **ref. *8***

11 Centrifuge at 110,000*g* in a Beckman SW28 rotor for 30 min at 4°C
12 Recover the major light-scattering band slightly more than halfway down the gradient (**Fig. 2**, SRZ) by use of a density gradient fractionator Layer the recovered band over quasiequilibrium sucrose gradients made from 5.6-mL layers of 300, 400, 500, and 600 mg/mL sucrose in maintenance buffer
13 Centrifuge for 1 h at 110,000*g* in a SW28 rotor at 4°C, and recover the light-scattering band (**Fig. 2**, SQE) Dilute the virus with an equal volume of maintenance buffer. Pellet at 90,000*g* in a Type 30 rotor for 30 min at 4°C Quickly aspirate the supernatant from the pellet and resuspend in 0 5–1 mL of maintenance buffer with a Pasteur pipet.
14 The pellets should resuspend easily, and highly purified preparations will have a milky appearance Optimum virus recovery is 2–5 $A_{260}$ U/100 g of tissue, but the purified virus preparations exhibit considerable light scattering because of the membrane and size of the virions Also, because the virions contain a low proportion of RNA (<2%), a prominent peak is not observed at 260 nm However, the yields may vary, depending on the age of the plants, the environmental conditions under which the plants were grown, and the variables introduced during preparation
15. Recovery of SYNV RNA is relatively straightforward by dissociation of virus by 1% sodium dodecyl sulfate (SDS) followed by sucrose density gradient centrifu-

gation (**Fig. 2**, RNA) This procedure also provides a sensitive estimate of the contamination, since ribosomes are the major host contaminants and they contain ~65% RNA, whereas SYNV has <2% RNA Also, a variety of phenol extraction methods can be used, but the yields are low and are somewhat variable

### 2.4. Notes on SYNV Purification

1 About 8 d after mechanical inoculation of leaves, *C quinoa* develops water-soaked lesions about 1 mm in diameter that turn tan-colored 2–3 d later Virus can be transferred from single lesions during the first 1–2 d after their appearance Upper uninoculated *C quinoa* leaves usually develop a mild systemic mottle in 3–4 wk
2 The infectivity of SYNV is lost quickly unless tissue is ground in the presence of a reducing agent, such as sodium sulfite or mercaptoethanol In the presence of 0.5% sodium sulfite, the infectivity of leaf extracts is maintained for more than 48 h at 4°C. Purified preparations of the virus stored at –80°C in maintenance buffer maintain their infectivity indefinitely
3 The interfaces and the layers used to make gradients form more easily if a glass pipet with a sealed tip and a 2- to 3-mm hole in the side just above the tip is used
4 The amount of tissue extracted can be increased if one has access to a large-volume zonal rotor for **step 5** Under these conditions, a larger Buchner funnel containing a proportionately increased surface area will be needed to accommodate the larger volume recovered from the interface between the 300 and 600 mg/mL sucrose layers
5 A particularly critical variable in the purification procedure is the thickness of the Celite pad used for filtration Pads thicker than 7 5 mm diminish yield of virus, but pads less than 2 5 mm result in filtrates contaminated with chloroplast fragments
6 Despite care in filtration, contamination with host components at this stage is variable, and some filtrates may contain traces of chlorophyll These contaminants are normally difficult to remove at this stage, because repeated filtration will result in loss of virus Moreover, a considerable quantity of the contaminating host material will sediment with the virus in subsequent stages of purification, so these preparations should be kept separate from virus preparations of higher purity However, the partially pure preparations can be used for purposes that require less purity

## 3. Polyribosome Purification from Tobacco

### 3.1. Introduction

Routine and reproducible procedures for isolation of polyribosomes from a variety of plant tissues was first accomplished by Jackson and Larkins *(9)* This procedure, which was initially developed to isolate polyribosomes from SYNV infected tobacco leaf tissue, relied in part on the use of EGTA (ethyleneglycol *bis*-[2 amino ethyl ether] tetra-acetic acid) for chelation of metals that cause polysomes to precipitate during the first low-speed centrifugation steps. The method subsequently proved to be adaptable to most tissues of tobacco and to a wide variety of different plant, fungal, and insect species

Because of its general utility for studies of the replication of SYNV ***(16,17)*** and its potential use in studies of other viruses, we have introduced this method here.

## 3.2. Materials

Buffers: All solutions are prepared in baked glassware using analytical grade reagents, and are autoclaved before using. Use ultrapure ribonuclease free sucrose and maintain RNase-free reagents by wearing gloves when handling them, and by using flamed or baked spatulas and autoclaved stir bars when preparing solutions. Add EGTA, β-mercaptoethanol, and Triton X-100 to appropriate buffers, just before use

1. EGTA stock: Add 38 g of EGTA to 85 mL of $H_2O$. Adjust pH to 8.0 with 5*M* NaOH and adjust the volume to 100 mL. Store at –20°C in 5-mL aliquots.
2. Triton X-100 (20%): Add 20 mL of reagent grade Triton X-100 to 80 mL of dd-$H_2O$. Autoclave, then mix occasionally, as the temperature cools, to prevent separation of the phases. Store at room temperature.
3. Extraction buffer: 200 m*M* Tris-HCl, pH 9.0, 400 m*M* KCl, 35 m*M* $MgCl_2$, 25 m*M* EGTA, and 200 m*M* sucrose and 1% Triton X-100. To prepare 2X extraction buffer, add 24.22 g Tris base, 29.82 g KCl, 7.10 g $MgCl_2$, and 68.46 g sucrose (RNase-free) to 450 mL of $H_2O$. Adjust the pH to 9.0 with HCl, and bring the volume to 500 mL. Store at –20°C indefinitely as 100-mL aliquots. Just before use, dilute the necessary volume of 2X extraction buffer with an equal volume of a dilution buffer containing 50 m*M* EGTA from the 1*M* stock solution, 2% β-mercaptoethanol, and 2% Triton X-100 diluted from the 20% stock (*see* **Subheading 3.4., Note 1**).
4. Sucrose pad buffer: 40 m*M* Tris-HCl, pH 9.0, 200 m*M* KCl, 35 m*M* $MgCl_2$, 5 m*M* EGTA, and 1.75*M* sucrose. Add 1.92 g Tris Base, 5.96 g KCl, 3.44 g $MgCl_2$ and 240 g sucrose to 450 mL of $H_2O$. Adjust the pH to 9.0 with HCl, and the volume to 500 mL. Store at –20°C in 50-mL aliquots. Add 250 µL of 1*M* EGTA to each 50-mL aliquot just before use.
5. Resuspension buffer: 40 m*M* Tris-HCl, pH 8.5, 200 m*M* KCl, 30 m*M* $MgCl_2$, and 5 m*M* EGTA. Add 0.48 g Tris base, 1.49 g KCl, and 2.03 g $MgCl_2$ to 95 mL of $H_2O$. Bring the pH to 8.5 with HCl. Store at –20°C in 12-mL aliquots. Add 60 µL of 1*M* EGTA (5 m*M*) just before use.
6. Sucrose gradient buffer: 40 m*M* Tris-HCl, pH 8.5, 20 m*M* KCl, and 10 m*M* $MgCl_2$. To make 10X stock sucrose gradient buffer, add 4.84 g Tris base, 1.49 g KCl, and 2.03 g of $MgCl_2$ to 95 mL of $H_2O$. Adjust the pH to 8.5 with HCl, and the volume to 100 mL. Store at –20°C in 5-mL aliquots.

## 3.3. Methods

### 3.3.1. Method for Polyribosome Extraction

1. Prechill all buffers, mortars, pestles, rotors, and centrifuges to 4°C (*see* **Subheading 3.4., Note 2**)

2 For isolation of polysomes from tobacco and similar dicots, collect leaves and remove the midribs Separate into eight batches, each of which consists of 2 5 g of leaf blades (*see* **Subheading 3.4., Note 3** for isolation of polyribosomes from cereals)

3a For isolation of total (free and membrane-bound) polysomes, immediately grind each batch for 2 min in 25 mL of cold 1X extraction buffer using a prechilled (~4°C) mortar and pestle Change to a fresh, cold mortar and pestle after two grinds Perform this procedure in a fume hood, because β-mercaptoethanol is noxious Pour each of eight homogenates into chilled 50-mL plastic or glass centrifuge tubes that can withstand *g*-forces of up to 25,000*g* Store on ice prior to centrifugation

3b. To separate membrane-bound polysomes from the free polysomes *(17)*, modify the extraction buffer used to grind the tissue by adding only 50 m*M* KCl (final concentration), and eliminate the Triton X-100. Then centrifuge the brei for 5 min at 500*g* in a preparative fixed-angle rotor (in a Sorval SS34 Rotor) to pellet nuclei, chloroplasts, mitochondria, and organelle fragments Discard the low-speed pellet and transfer the supernatant into eight fresh plastic or glass tubes Then centrifuge at 20,000*g* in the SS34 rotor to separate the supernatant fraction (free polysomes) from the pellet (membrane-bound polysomes). Save both the supernatant and pellet fractions. Add KCl to 400 m*M* and Triton X-100 to 1% to the supernatant Resuspend the pellet containing the membrane-bound polysomes in the same volume of extraction buffer (containing 400 m*M* KCl and 1% Triton X-100) as the free polysome supernatant fraction

4 Centrifuge the samples from **steps 3a** (total polysomes) or **3b** (supernatant = free polysomes, pellet = membrane-bound polysomes) at 20,000*g* for 10 min at 4°C

5 While the samples in **step 4** are spinning, pipet 5 mL of sucrose pad buffer into 30-mL Beckman polycarbonate bottles Chill on ice Remove the cleared supernatant from the cell debris pellet carefully with a 25-mL disposable plastic pipet Load the supernatant over the sucrose pad, being careful not to disturb the interface

6. Pellet the polysomes by centrifuging at 315,000*g* in a fixed-angle ultracentrifuge rotor (Beckman Ti60 rotor) for 80 min at 4°C (*see* **Subheading 3.4., Note 4**)

7 Remove and discard the supernatant by aspirating off the green material Then, rinse the sides of the tubes with 5 mL of ice-cold dd$H_2O$ Remove the rinse water by aspiration, then repeat the rinsing procedure Aspirate the sucrose pad off of the pellet and invert the tube to expose the clear polysome pellet Quickly rinse the pellet with 1 mL of cold $H_2O$ to remove residual sucrose. To do this, turn the tube so that the side containing the pellet is on top Add 1 mL of $H_2O$ and rotate the tube in a complete circle during a 15-s interval Resuspend each pellet in 1 25 mL of 1X resuspension buffer by dislodging them with a Pasteur pipet and vortexing. Leave on ice for 15 min Combine the suspensions, then rinse each tube sequentially with 1 25 mL of 1X resuspension buffer

8 Measure the optical density at $A_{260}$ to determine the yield (*see* **Subheading 3.4., Note 5**)

9 Transfer the suspensions to glass or polypropylene tubes. Aliquots (~100 µL) of the resuspended polyribosomes may be pipeted into liquid nitrogen and the frozen spheres may be stored in sealed vials indefinitely These polysomes may be used for in vitro translation, RNA extraction, or for fractionation over sucrose gradients (*see* **Subheading 3.4., Note 6**)

### 3.3.2. Method for Sucrose Gradient Fractionation

This procedure allows visualization of the extent of polysome polymerization and permits recovery of different size classes of polysomes. The polysomes are separated by centrifugation over sucrose gradients, as described below, to separate the polysomes according to the number of ribosomes on the mRNA molecule. **Figure 3** shows the nearly identical profiles of free and membrane-bound polysomes from uninfected and SYNV-infected tobacco leaves, and gives some indication of typical profiles recovered from this tissue. The gradients are analyzed in a density gradient fractionator that can monitor the absorbance of the eluate at 254 nm. For analysis, it is recommended that two duplicate samples are run To prepare samples for the gradients, use 100 µL of polysomes in 1X resuspension buffer. Add 30 µL of proteinase K solution (20 mg/mL) and incubate the samples on ice for 15 min. Proteinase K treatment reduces aggregation of polyribosomes, which are sometimes formed by interactions of nascent polypeptides, to give a more reliable estimate of the polysome size distribution than would be obtained with untreated samples

1 Sucrose gradient preparation Prepare gradients for 18–48 h before they are needed, to permit equilibration Protect the solutions from RNase contamination by using flamed glass pipets with a sealed tip and a small hole in the side to form the gradient layers Normally, use a Beckman SW 41 rotor with six buckets that hold 12-mL tubes (14 × 89 mm) The sucrose layers contain 2 mL of 150 mg/mL (layer A), 4 mL of 300 mg/mL (layer B), 4 mL of 450 mg/mL (layer C), and 2 mL of 600 mg/mL of 1X gradient buffer (layer D) Load layer D on the bottom, then load the C, B, and A layers, consecutively.
2 Let the gradients equilibrate at 4°C for at least 18 h before use.
3 Load 0 5 mL samples containing 2.5–6 $A_{260}$ units of polysomes on top of the equilibrated gradients and centrifuge at 235,000*g* for 70 min at 4°C
4 For larger scale separations, use a SW 27 rotor with 25 × 89-mm tubes. Form a 36-mL gradient with 6 mL of 150 mg/mL, 12 mL of 300 mg/mL, 12 mL of 450 mg/mL, and 6 mL of 600 mg/mL. Layer 1-mL samples containing 10–20 $A_{260}$ units on top of the gradients. Centrifuge at 110,000*g* for 2 h 20 min at 4°C.
5 For analytical use, and to conserve sample size, use a SW 50 1 rotor with 13 × 51-mm tubes. The 4 8-mL gradients should be composed of 0 8, 1.6, 1 6, and 0.8 mL of 150 mg/mL, 300 mg/mL, 450 mg/mL, and 600 mg/mL, respectively The sample size should be 0.2 mL with 1–2 $A_{260}$ units. Centrifuge at 245,000*g* for 45 min at 4°C Alternatively, use a SW 60Ti rotor with 11 × 60-mm tubes containing

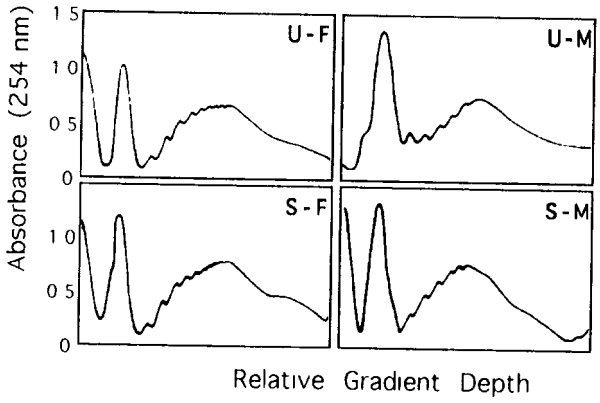



Fig 3 Sucrose density gradient analysis of free and membrane-bound polyribosomes isolated from uninfected and SYNV-infected tobacco. The panels designated U-F, U-M, S-F, and S-M refer to free and membrane-bound polysomes isolated from leaves of uninfected and SYNV-infected plants, respectively. Although the profiles vary slightly, there is no major correlation between the number of monomers associated with the different classes of polysomes. However, we have shown ***(16,17)*** that about 2–5% of the messenger RNA in SYNV-infected plants is viral-specific The free polysomes contain sequences hybridizing to nearly 100% of the viral RNA, but the RNA derived from membrane-bound polysomes hybridizes to only 40% of the RNA These results suggest that a specific subset of the viral mRNAs are membrane-associated. Adapted from **ref. *17***

0.66, 1 34, 1 34, and 0 66 mL layers of sucrose, as described above The sample size should also be 0 2 mL with 1–2 $A_{260}$ units After centrifugation at 335,000*g* for 30 min at 4°C, the gradients should be fractionated in a density gradient fractionator attached to a UV monitor at 254 nm

## 3.4. Notes on Polysome Procedure

1 The recovery of polyribosomes is critically dependent on maintaining the correct ratios of EGTA to $Mg^{2+}$ in the solutions. Because of metals found in vacuoles, polyribosomes precipitate when insufficient amounts of EGTA are present However, when the ratio of $Mg^{2+}$ to EGTA is too low, the polyribosome subunits dissociate (*see* **ref. *9*** for a discussion of these variables).

2 Maintaining the temperature close to 4°C throughout the various steps is important for polysome stability Minor amounts of RNase will quickly degrade polysomes, and, although the relatively high pH and ionic strength of the buffers mediate against RNase activity, transient increases in the temperature can affect the extent of polymerization observed in the polysome profiles.

3 For cereals ***(18)***, reduce the extraction buffer to 2 mL for each gram of leaf tissue The relative proportion of vascular tissue is much higher in cereals than in dicots and this tissue cannot be easily removed This results in lower recovery of cytosol from the cereal tissue

4 To avoid polycarbonate tube breakage and subsequent run failure at these high gravational forces, always wash these alkali-sensitive tubes in very mild detergent approved for this purpose Reuse the tubes only three times, and, before each centrifuge run, pipet ~100 µL of $H_2O$ into the rotor receptacles before inserting the centrifuge tubes This provides a cushion that helps create a uniform force around the base of the tubes during centrifugation The cushion disperses the gravitational stress uniformly around the tube and reduces tube cracking and sample loss.

5 The yield of polyribosomes is 0.75 mg/g fresh weight of young tobacco leaves, assuming an extinction coefficient of ~15 $A_{260}$ U/mg for ribosomes containing ~65% RNA

6. Variations of the extraction procedure have been used for recovery of polyribosomes from many different tissues and species of plants, from fungi and from insects. The procedure thus has broad applicability that extends beyond its use in plants

## 4. Preparation of Replicase Extracts from Nuclei of SYNV-infected Tobacco

### *4.1. Introduction*

An indispensable tool in studies of the replication of rhabdoviruses and other negative-strand viruses has been the use of in vitro polymerases for the analysis of the factors that contribute to transcription of the viral messenger RNAs and to the replication of the genomic RNAs. These studies have been especially important in our understanding of the biochemistry and regulation of the replication processes of vesicular stomatitis virus *(19)*. However, purified SYNV virions lack an active polymerase, and this has hampered research. Wagner et al. *(10)* have recently circumvented this problem by devising a procedure for isolating polymerase activity from the nuclei of infected tobacco leaves. We hope this procedure will be of general utility for studies of other nuclear associated rhabdoviruses

### *4.2. Materials*

1 Nuclei extraction buffer: Mix 40% v/v glycerol, 600 m*M* ribonuclease-free sucrose, 5 m*M* $MgCl_2$ Adjust the mixture to 480 mL with $ddH_2O$. Then, add 1 mL of diethylpyrocarbonate (DEPC), mixed with 1 mL of ethanol, to destroy RNase activity. **Caution:** DEPC is toxic, so carry out this step in a hood (*see* **Subheading 4.4., Note 1**) Let the DEPC-treated $H_2O$ sit overnight at 37°C, and autoclave to destroy the DEPC. Store at 4°C. Shortly before use, add Tris-HCl, pH 8 0, to 25 m*M*, spermine to 2 m*M*, and β-mercaptoethanol to 10 m*M* Immediately (<5 min) before use, add phenylmethane sulfonyl fluoride (PMSF) to 1 m*M* and add $H_2O$ to 500 mL The PMSF stock is stored at room temperature as a 200-m*M* solution in isopropanol

2. Mannitol buffer. For a 500-mL solution, add 250 m*M* mannitol (22 9 g), 5 m*M* $MgCl_2$ (0 51 g) and $H_2O$ to 480 mL. Add 0 5 mL DEPC, leave at 37°C overnight,

autoclave, and equilibrate the buffer to 4°C. Add Tris-HCl, pH 8 0, to 25 m*M* and β-mercaptoethanol to 10 m*M* Add PMSF to 1 m*M* immediately before use and adjust the volume to 500 mL

3 95% Percoll stock solution This solution contains 95% Percoll (Pharmacia, Uppsala, Sweden)/5% mannitol buffer Prepare the 95% Percoll stock solution in a sterile beaker by mixing 70 mL of Percoll, 1 842 mL of 1*M* Tris-HCl, pH 8 0, 368 µL of 1*M* $MgCl_2$, and 3 37 g of mannitol Remove 19 75 mL for the 75% Percoll stock solution Just before use, add 57 5 µL β-mercaptoethanol and 216 µL of 200 m*M* PMSF stock solution Do not autoclave or filter the Percoll containing buffers

4 75% Percoll stock solution Make a 25-mL solution containing 75% Percoll/25% mannitol buffer To prepare the 75% Percoll buffer, mix 19 75 mL of the 95% Percoll stock solution from **step 3** and dilute with 5 25 mL of mannitol buffer containing 10 m*M* β-mercaptoethanol and 1 m*M* PMSF added immediately before use

5 Polymerase elution buffer Prepare a 5-mL solution containing 40% v/v glycerol, 200 m*M* $(NH_4)_2SO_4$, 25 m*M* hydroxyethylpiperazine ethane sulfonic acid (HEPES, pH 8 0), 5 m*M* $MgCl_2$ Immediately before use, add dithiothreitol (DTT) to 3 m*M*, Aprotinin and Pepstatin to 1 µg/mL each, Leupeptin to 0 5 µg/mL, and PMSF to 1 m*M*

## 4.3. Methods

### 4.3.1. Method for Isolation of SYNV Polymerase

1 Harvest 100 g of SYNV-infected *N edwardsonii* leaf tissue displaying netting symptoms at 10–14 d postinoculation *(7)*. Tissue frozen at –80°C may be stored for several months without detectable loss in polymerase activity

2. Grind tissue in liquid nitrogen in a stainless steel blender Using a reostat, start the blender at medium speed, and add some liquid nitrogen Then add the frozen tissue, hold the cover top down with an asbestos glove, and grind the tissue at the highest setting in three 30-s bursts Lower the speed to a medium setting, and remove the top, letting the remaining nitrogen evaporate Do not stop the blender between the bursts or until the nitrogen evaporates, because it will freeze up and you will not be able to start it again

3. Quickly transfer the ground tissue into an Omnimixer (Dupont, Wilmington, DE) Add 400 mL of chilled nuclei extraction buffer, and mix at the highest setting to get a uniform slurry **Step 3** and all subsequent isolation steps are performed at 4°C.

4 Put the slurry in a 600-mL beaker containing a stirbar While the solution stirs, add Nonidet P-40 (NP40) to 0 6%, and stir for 5 min

5 Pour the cold slurry through autoclaved 350-µm, 62-µm, then 44-µm nylon mesh filters This is accomplished by attaching the meshes to a 2-L plastic beaker with rubber bands. First, put a course mesh net on the bottom, then add the fine mesh filter, the middle mesh, and another course mesh net on the top

6 Filter the slurry by gravity flow. Then remove one mesh at a time and squeeze the remaining liquid into the beaker Save this filtrate Next, scrape the material from

the course mesh back into the omnimixer and mix with the remaining 100 mL of nuclei extraction buffer

7. Add NP40 to 0 6%, and filter this slurry through the meshes Combine this with the first filtered slurry
8. Transfer the filtered slurry to 250-mL centrifuge bottles and centrifuge for 10 min at 4000*g* in a Sorvall GSA rotor If the detergent has worked as expected, a dark-green mass of disrupted chloroplast fragments should float to the top of the bottles The pellet will contain the nuclei and will be a grayish green Using a vacuum, remove the top layer carefully, then carefully aspirate the buffer from the pellet Wipe the sides of the bottles with kimwipes to remove the rest of the dark-green supernatant
9. Gently resuspend the pellets in 95% Percoll stock solution and distribute to four 30 mL silanized Corex tubes (we use silanized tubes, because the mixtures tend to stick to the surface of unsilanized glass) Bring each tube to ~15 mL with the 95% Percoll stock, gently mix, then gently pipet a 10-mL layer of mannitol buffer over the resuspended Percoll pellets.
10. Centrifuge at 4°C for 10 min at 4000*g* in a Sorvall HB-4 swinging-bucket rotor
11. Collect nuclei from the mannitol buffer/95% Percoll interface in each tube The material at this interface should be light-green and very viscous Use a bent-tip 15-gage, or larger, diameter needle, or a similar wide-bore device, attached to a syringe, to collect the viscous band
12. Distribute the nuclei to two fresh 30-mL glass tubes, and dilute the nuclei in each tube to 15 mL with mannitol buffer After dilution, the concentration of Percoll should be less than 50%
13. Underlayer 10 mL of the 75% Percoll stock solution to make a cushion on the bottom of each tube
14. Centrifuge for 5 min at 4000*g* in the HB-4 rotor
15. Collect the nuclei from the interface and the top half of the 75% Percoll layer and pool them in one fresh 30-mL tube (*see* **Subheading 4.4., Note 2**)
16. Dilute the nuclei to 25 mL with Mannitol buffer, and centrifuge for 5 min at 1000*g* in the HB-4 rotor Aspirate the supernatant from the nuclear pellet. At this stage, the very loose pellet will be about 1–2 mL Shake the pellet gently to resuspend the nuclei
17. Transfer the nuclei from the pellet to 1 5-mL Eppendorf centrifuge tubes in 500-µL batches, using a wide-bore instrument (e.g , a Pipetteman P-1000 with a cutoff tip) If the pellet is too viscous to pipet, adding a small amount (~1–2 mL) of mannitol buffer will facilitate the transfer
18. To recover the polymerase from the nuclei, add 500 µL of nuclei to 1 5-mL Eppendorf centrifuge tubes, and an equal volume of polymerase elution buffer to each tube Then, add 50 U of RNasin (Promega) to each tube Rock the tubes gently for 30 min at 4°C.
19. Centrifuge the tubes for 30 min at 16,000*g* in an Eppendorf microcentrifuge at 4°C.
20. Collect the supernatants from the tubes, pool, and mix the supernatants and store as 200-µL aliquots Freeze the polymerase extract in liquid nitrogen and store at

–80°C Usually, 3–5 mL of SYNV polymerase extract is obtained from 100 g of tissue using this protocol

### 4 3.2. Polymerase Reactions

1 For each reaction mixture, prepare 20 µL of the polymerase extract in a 200-µL reaction. The final concentration of the reactants is 6 m*M* $MgCl_2$, 50 m*M* $(NH_4)_2SO_2$, 12.5 m*M* HEPES, pH 8 0, 2 m*M* DTT, 1 m*M* ATP, 0 5 m*M* CTP, 0.5 m*M* GTP, and 20 µ*M* UTP, plus 50 µCi of [$\alpha^{32}$P] UTP, 5 U of DNase I, 50 U of RNasin (Promega), and 2% v/v glycerol.
2 Incubate the reactions at 28°C for various periods (*see* **Subheading 4.4., Note 3**)
3 Stop the reactions by adding SDS to 0 5% and EDTA to 5 m*M* React for 30 min at 42°C with proteinase K (500 µg/mL) to digest the proteins.
4 Extract the RNAs with phenol-chloroform and precipitate by adding 200 µL of 5*M* $NH_4$Acetate and 1 mL of ethanol to 200 µL of extracted RNA
5 **Figure 4** shows the results of a slot blot hybridization obtained from transcriptions with purified nuclei and extracted polymerase from uninfected and SYNV-infected plants (*see* **Subheading 4.4., Note 4**)

## 4.4. Notes

1 **Caution:** DEPC is volatile, so a hood should be used at all times DEPC can be extremely irritating to the eyes, mucous membranes, and skin when used without ventilation DEPC can also cause loss of sensation in the fingers and outer extremities when working in areas where high concentrations are allowed to accumulate The compound is also suspected of being a carcinogen Therefore, it should always be used in a hood DEPC reacts with primary amines and also inactivates both proteins and nucleic acids, so direct contact with the agent can destroy the biological or biochemical activity of enzymes and single-stranded nucleic acids Upon autoclaving, DEPC decomposes to yield ethanol and $CO_2$. Mixture with ethanol increases the solubility in $H_2O$, and a 50% solution disperses much more readily in solutions than concentrated DEPC.
2 Most of the nuclei pellet through the 75% Percoll, but the SYNV transcriptase activity in the pelleted nuclei is far lower than that of the nuclei from the 75% interface From this result, we believe that the nuclei containing the highest amounts of polymerase activity have lower densities than nuclei derived from cells that do not contain actively replicating virus
3. The polyadenylated leader RNA, and full-length polyadenylated N, M2, sc4, M, and G mRNAs appear in the order of their location on the SYNV genome By 6 h postinoculation, full-length polyadenylated mRNA products can be detected by selection with oligo(dT) cellulose chromatography
4. We have also analyzed the polymerase RNA products by a variety of other procedures that are described in Wagner et al. ***(10)*** and Wagner and Jackson ***(20)***

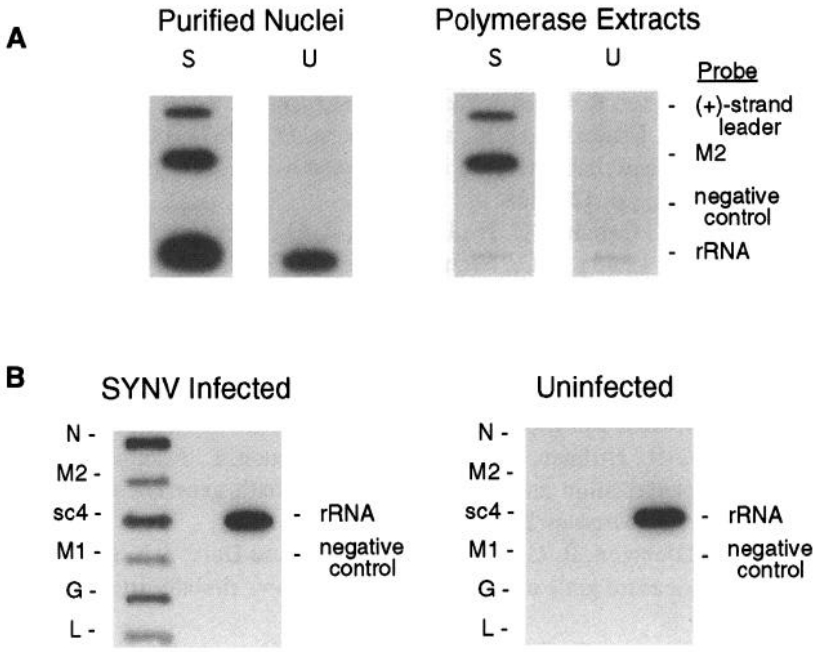



Fig. 4. Slot-blot hybridization of RNA synthesized in reactions containing purified tobacco nuclei and polymerase extracts from nuclei. For this experiment, unlabeled DNA probes representative of various parts of the viral genome, or to a ribosomal DNA clone from plants, were placed in individual slots and filtered onto a nitrocellulose membrane support. The DNA was bound to the membrane, and radioactive RNA products synthesized in 30-min polymerase reactions were used for hybridization. The probes were derived from the SYNV (+)-strand leader RNA, the N, M2, sc4, M1, G, and L genes, and a ribosomal RNA control. **(A)** Illustrates the hybridization of RNA products from purified nuclei and polymerase extracts derived from SYNV-infected tissue (S) and from uninfected plants (U). The results show that nuclei from SYNV-infected plants actively synthesize abundant amounts of the SYNV leader RNA and the M2 RNA, as well as host rRNA; the nuclei from uninfected plants synthesize only the host rRNAs. In contrast, the polymerase activity eluted from the nuclei is specific for the SYNV RNA. **(B)** Shows that nuclei from SYNV-infected plants synthesize each of the SYNV genes and rRNA, and verify that nuclei from uninfected plants synthesize only rRNA.

## Acknowledgments

We thank Drs. Diane Lawrence, Teresa Rubio, and Michael Goodin for reading the manuscript. We also thank Drs. Lawrence and Rubio for assistance with the figures. This research was supported by grants to A. O. J. from the National Science Foundation extending from 1978 to the present award, DMB 94-18086.

## References


1 Wagner, R. R (1987) Rhabdovirus biology and infection an overview, in *The Rhabdoviruses* (Wagner, R R , ed ), Plenum, New York, pp 9–74

2 Jackson, A O , Francki, R I B , and Zuidema, D (1987) Biology, structure and replication of plant rhabdoviruses, in *The Rhabdoviruses* (Wagner, R R., ed ), Plenum, New York, pp 427–508

3 Wunner, W H , Calisher, C H , Dietzgen, R G , Jackson, A O , Kitajama, E W , Lafon, M , Leong, J C , Nichol, S , Peters, D , Smith, J S , and Walker, P J (1995) Family rhabdoviridae, in *Virus Taxonomy Classification and Nomenclature of Viruses, Sixth Report of the International Committee on Taxonomy of Viruses* (Murphy, F. A , Fauquet, C M , Bishop, D H L , Ghabrial, S A , Jarvis, A W , Martelli, G P , Mayo, M A , and Summers, M D , eds ), Springer-Verlag, New York, pp 275–288

4 Scholthof, K.-B., Hillman, B I , Modrell, B , Heaton, L A , and Jackson, A O (1994) Characterization and detection of sc4 a sixth gene encoded by sonchus yellow net virus *Virology* **204,** 279–288.

5 Wetzel, T , Dietzgen, R G , Geering, A D W , and Dale, J L (1994) Analysis of the nucleocapsid gene of lettuce necrotic yellows rhabdovirus *Virology* **202,** 1054–1057

6 Wetzel, T , Dietzgen, R G , and Dale, J L (1994) Genome organization of lettuce necrotic yellows virus *Virology* **200,** 401–412

7 Francki, R I B , Randles, J W , and Dietzgen, R G (1989) Lettuce necrotic yellows virus, in *AAB Descriptions of Plant Viruses* No 343

8 Jackson, A O. and Christie, S R (1977) Purification and some physicochemical properties of sonchus yellow net virus *Virology* **77,** 344–355.

9 Jackson, A O and Larkins, B A (1976) Influence of ionic strength, pH, and chelation of divalent metals on isolation of polyribosomes from tobacco *Plant Physiol* **51,** 5–10.

10. Wagner, J D O , Choi, T -J , and Jackson, A O (1996) Extraction of nuclei from sonchus yellow net rhabdovirus infected plants yields a polymerase that synthesizes viral mRNAs and polyadenylated plus-strand leader RNA *J Virol* **70,** 468–477

11. Hunter, B. G., Richardson, J , Dietzgen, R. G , Karu, A , Sylvester, E S , Jackson, A O , and Morris, T. J (1990) Purification and characterization of strawberry crinkle virus *Phytopathology* **80,** 282–287

12 Black, L M (1979) Vector monolayers and plant viruses *Adv Virus Res* **25,** 192–271

13. Adam, G., Heegard, P , Bog-Hansen, T C., and Mundry, K W (1987) Lectins as probes for the assay of rhabdovirus infections in plants *J Virol Methods* **17,** 263–275

14 Francki, R I B (1973) Plant rhabdoviruses *Adv Virus Res* **18,** 257–345

15 Jones, R. W and Jackson, A O (1990) Replication of sonchus yellow net virus in infected protoplasts *Virology* **179,** 815–820

16 Milner, J J. and Jackson, A O (1979) Sequence complementarity of sonchus yellow net virus RNA with RNA isolated from the polyribosomes of infected tobacco *Virology* **97,** 90–99
17 Milner, J J , Hakkaart, M J J , and Jackson, A O (1979) Subcellular distribution of RNA sequences complementary to sonchus yellow net virus *Virology* **98,** 497–501
18. Gustafson, G. D , Larkins, B A , and Jackson, A O (1981) Comparative analysis of polypeptides synthesized *in vivo* and *in vitro* by two strains of barley stripe mosaic virus *Virology* **111,** 579–587.
19 Banerjee, A. K and Barik, S (1992) Gene expression of vesicular stomatitis virus genome RNA *Virology* **188,** 417–428
20 Wagner, J D O and Jackson, A O (1997) Characterization of the components and activity of sonchdus yellow net rhabdovirus polymerase *J Virol* **71,** 2371–2382

# 8

# Hordeivirus Isolation and RNA Extraction

**Diane M. Lawrence and Andrew O. Jackson**

## 1. Introduction

The hordeivirus group of RNA viruses contains three members: barley stripe mosaic virus (BSMV), poa semilatent virus (PSLV), and lychnis ringspot virus (LRSV), with anthoxanthum latent bleaching virus (ALBV) considered to be a possible fourth member *(1)*. Hordeiviruses have a diverse host range; BSMV, PSLV, and ALBV primarily infect members of the graminae, and LRSV is known to infect several members of the dicots. As yet, no vectors have been identified that are capable of transmitting hordeiviruses. BSMV and LRSV are transmitted through seed and pollen, with the efficiency being dependent on many factors, including the virus strain, the host plant, and environmental factors. However, to date, PSLV and ALBV have not been shown to be seed-transmitted.

Hordeiviruses are tripartite viruses containing a positive-sense single-stranded RNA genome. Each genome is encapsidated within a rod-shaped (100–160 nm in length) particle that is comprised of a single-capsid protein species (**Fig. 1**). Extensive studies have been performed on the type member of the group, BSMV, and therefore we will focus on this member throughout this chapter.

The three genomes of BSMV are designated α, β, and γ The α and β genomes are 3.8 and 3 3 kb in size, respectively, but the γ genome differs in size depending on the strain; for example, the ND 18 strain is 2.8 kb, and the type strain is 3.2 kb in size. The complete nucleotide sequences of the α, β, and γ genomes of the type strain of BSMV have been known for several years *(2–4)*. The availability of infectious transcripts has permitted mutational analyses and dissection of the viral genome, to further our understanding of the BSMV infection process.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor ©Humana Press Inc , Totowa, NJ

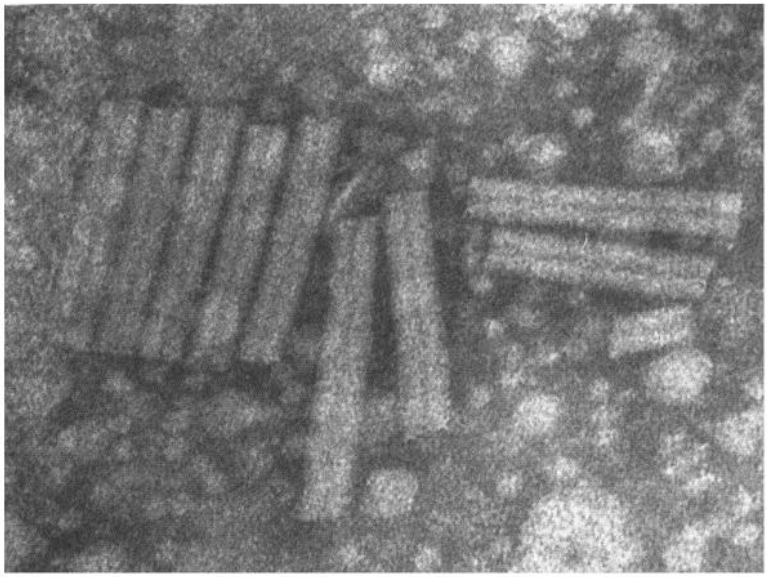


Fig. 1. Hordeivirus morphology. Electron micrograph of negatively stained particles of BSMV. The virus particles are ~25 nm in width and vary in length from ~100 to 160 nm.

The genome organization of BSMV is shown in **Fig. 2**. All three RNAs have a 7-methylguanosine cap at the 5' terminus and a conserved 3' region containing an internal poly(A) tail directly following the stop codon of the 3' proximal open reading frame (ORF). Following the poly(A) tail is a 238 nucleotide tRNA-like structure that is capable of binding tyrosine in vitro. The α, β, and γ genomes are required to establish a systemic infection in plants; the α and γ genomes are sufficient for viral RNA replication in protoplasts ***(5)***. The α genome encodes a single 130-kDa protein (αa) that contains both methyl transferase- and nucleotide-binding domains. At least four proteins are encoded by the β genome: βa (23 kDa) is followed by an intergenic region that precedes a series of overlapping genes, designated the triple gene block. The triple gene block encodes proteins of 58 kDa (βb), 14 kDa (βd), and 17 kDa (βc). The βa protein is the BSMV capsid protein, but, despite its role in formation of virus particles, it is not essential for systemic spread ***(6)***. βb is known to bind RNA and nucleotide triphosphates in vitro. Hence it is speculated that it has a role as an RNA helicase in vivo, even though in vitro helicase activity has not been associated with the purified protein ***(7)***. βd and βc are both hydrophobic proteins, and immunolocalization of βd in infected barley tissue has shown that it

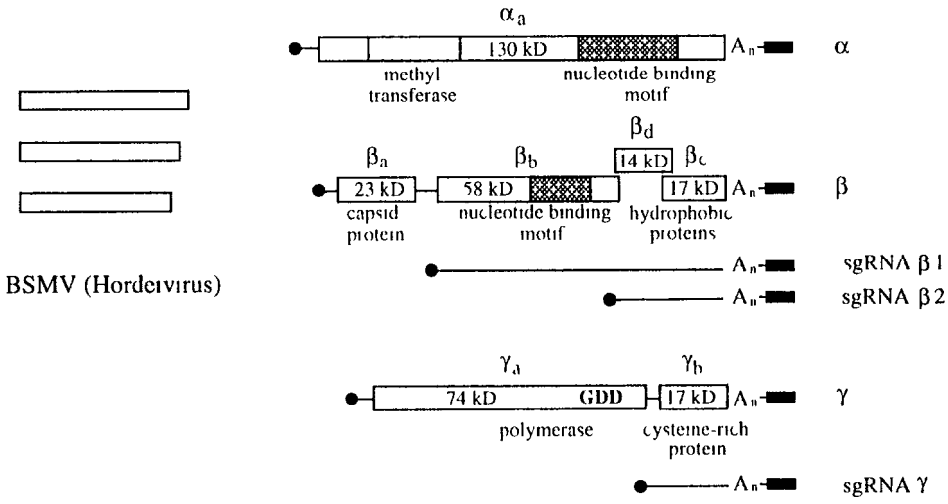



Fig 2 Genetic organization of BSMV. The filled circles and rectangles represent a cap structure and a tRNA-like structure, respectively. The sgRNAs utilized for expression of the 3' proximal genes are depicted directly beneath the genomes

is associated with the cell wall and membrane fractions ***(8)***. Mutational analysis of the triple gene block has shown that βb, βd, and βc are each essential for systemic infectivity in barley ***(6)***. It is postulated that βb, βd, and βc are expressed in vivo from two subgenomic (sg) RNAs that are 2.5 and 0.96 kb in size ***(9)*** When in vitro generated transcripts of the 3 3 kb genomic RNA and the 2.5 kb sgRNA containing authentic 5' termini were used to program in vitro translation reactions, the coat protein, and the βb protein, respectively, were detected. However, the 0.96 kb RNA served as an mRNA for synthesis of the βd protein, minor amounts of a translational readthrough product, βd', and βc ***(9)***

The γ genome encodes two proteins of 74 kDa (γa) and 17 kDa (γb) in size The γa protein contains the GDD domain present in polymerase proteins of single-stranded positive-sense RNA viruses, and is strictly required, in concert with αa, for viral replication ***(10)***. The γb protein is cysteine-rich and is expressed from a sgRNA ***(11)***. γb is known to affect virulence and expression of genes encoded by RNAβ; however, its biochemical function in infection is unclear at this time. It has been demonstrated that γb protein can bind nucleic acid ***(12)*** and that deletion of this gene attenuates viral replication; mutations in the cysteine-rich domain affect the symptom phenotype in barley ***(13)*** Thus far, all the proteins encoded by BSMV, except for βc, have been detected in infected barley tissue during infection.

**Table 1**
**Preparation of Sucrose Solutions for 6–24% (w/v) Sucrose Gradients**

| | % (w/v) Sucrose solutions | | | |
|---|---|---|---|---|
| | 6% | 12% | 18% | 24% |
| 60% (w/v) Sucrose | 2 5 mL | 10 mL | 15 mL | 20 mL |
| 200 m*M* Potassium phosphate buffer, pH 6 8 | 12 5 mL | 25 mL | 25 mL | 25 mL |
| 20% (v/v) Triton X-100 | 125 µL | 250 µL | 250 µL | 250 µL |
| $H_2O$ | 9 875 mL | 14 75 mL | 9 75 mL | 4 75 mL |

## 2. Materials

1 Borate buffer 0 5*M* boric acid ($H_3BO_3$), bring to pH 9 0 with NaOH Store at room temperature indefinitely Chill the buffer to 4°C prior to virus purification
2 20% (v/v) Triton X-100 Add 20 mL of Triton X-100 to 80 mL of $H_2O$ and autoclave for 5 min When the solution cools to approx 60°C, swirl intermittently to prevent two phases from forming Store at 4°C indefinitely
3 Potassium phosphate buffer Mix equal quantities of 200 m*M* solutions of $KH_2PO_4$ and $K_2HPO_4$ and store at 4°C The pH of this solution should be 6 8 Dilute 200 m*M* potassium phosphate to obtain 50 m*M* and 10 m*M* potassium phosphate buffers and store at 4°C
4 60% (w/v) Sucrose Dissolve 60 g sucrose to 100 mL with $H_2O$, store frozen or at 4°C.
5 20% (w/v) Sucrose pad Mix 30 mL of 60% (w/v) sucrose, 54 mL of 0 5*M* borate buffer, pH 9.0, 4.5 mL of 20% (v/v) Triton X-100 and 1 5 mL of $H_2O$
6 6–24% (w/v) Sucrose gradients Prepare 24, 18, 12, and 6% (w/v) sucrose solutions (*see* **Table 1**)
7 Proteinase K (2 mg/mL) in $H_2O$ Store at –20°C.
8 Bentonite, prepared as described by Fraenkel-Conrat et al ***(14)*** Stir 5 g of bentonite in 100 mL of $H_2O$, making sure that the bentonite is suspended well Centrifuge at 600*g* for 10 min. Recover the supernatant and recentrifuge at 8000*g* for 20 min. Resuspend the pellet in 100 mL of 100 m*M* disodium ethylenediaminetetraacetate (EDTA), pH 8 0 Break the pellet up well with a glass rod and stir for 48 h at room temperature Perform the two centrifuge steps as described previously Resuspend the pellet obtained following the second centrifuge step in 100 mL of 10 m*M* sodium acetate, pH 6 0 Break the pellet up and stir overnight at room temperature. Centrifuge at 8000*g* for 20 min Resuspend the pellet in 10 mL of 10 m*M* sodium acetate, pH 6 0. Determine the concentration of bentonite by adding 1.0 mL of bentonite to a preweighed weighing boat Dry off the liquid in an oven at 60°C overnight and determine the weight of the bentonite Dilute the bentonite to 10 mg/mL with 10 m*M* sodium acetate, pH 6 0 Store the bentonite stock at –20°C.

9 Ammonium carbonate buffer (pH 9 0). 200 m*M* ammonium carbonate, 2% (w/v) sodium dodecyl sulfate (SDS), 2 m*M* disodium EDTA and 200 mg/mL bentonite
10. Kirby's phenol Take a 500-g bottle of crystalline phenol and add 50 mL m-cresol, 0 5 g 8-OH quinoline, and 0 1*M* Tris-HCl, pH 8 8, to saturation Store in a dark bottle at 4°C.
11. Phenol·chloroform (1 1) Mix equal volumes of Kirby's phenol and chloroform and store in a dark bottle at 4°C
12 3*M* sodium acetate, pH 4 8 24 6 g sodium acetate in 75 mL $H_2O$, pH to 4 8 with glacial acetic acid, and make up to 100 mL with $H_2O$
13 TAE 40 m*M* Tris-acetate, pH 7 5, 1 m*M* EDTA 20X stock solution 96 8 g Tris base, 22 8 mL glacial acetic acid, 40 mL 0 5*M* EDTA in 1 L Dilute to 1X prior to use

## 3. Methods

### 3.1. Virus Purification

Extensive work on hordeivirus purification was initially carried out by Myron Brakke The purification described here is based on this methodology and incorporates minor modifications to the method described by Jackson and Brakke *(15)*

1 Collect BSMV-infected plant tissue, approx 7–10 d postinoculation Use immediately, or store at 4°C for up to a week (*see* **Note 1**)
2 Cut the leaves into approx 2-cm pieces and extract in borate buffer (300 g tissue 300 mL buffer) Usually 100 g of tissue are blended in 300 mL of buffer in a Waring blender at high speed for 2 min, or until the tissue is ground into a fine slurry
3 Squeeze the extract through several layers of cheesecloth to remove the macerated pulp
4. The filtrate is returned to the blender, another 100 g of tissue are added, and the extraction is repeated. The resulting filtrate is then used for a third round of extraction The final volume of the filtrate should be 350–400 mL
5 Centrifuge the filtrate at 3000*g* for 10 min
6 Remove the supernatant and measure the volume. Then add 0.05 vol of 20% (v/v) Triton X-100 to the supernatant and stir for 10 min at room temperature to dissociate membranes and chloroplast material
7 Add 7 mL of the 20% (w/v) sucrose pad to twelve 30-mL polycarbonate centrifuge tubes Carefully layer the tissue extract over the sucrose pad, causing as little disturbance to the sucrose pad as possible Centrifuge at 180,000*g* for 1 h at 4°C in a fixed-angle rotor
8 Decant the supernatants, including the sucrose pad, and resuspend each pellet in 4 5 mL of 50 m*M* potassium phosphate buffer, pH 6.8 To ensure that the pellets are suspended, a glass homogenizer is used to disperse the insoluble material into a fine suspension Combine the resuspended pellets in a beaker and stir at 4°C for 30 min

9. Centrifuge the resuspended pellets at 2000*g* at 4°C for 10 min Remove the supernatant. Be careful not to transfer any particulate matter from the pellet and adjust the volume to 57 mL with 50 m*M* phosphate buffer, pH 6 8. Add 3 mL of 20% (v/v) Triton X-100 and stir at 4°C for 10 min.
10 Prepare six 25-mL discontinuous step gradients in 25 × 89-mm centrifuge tubes These gradients can be prepared just prior to use. Carefully layer 7 mL 24% (w/v) sucrose, 7 mL 18% (w/v) sucrose, 7 mL 12% (w/v) sucrose, and 4 mL 6% (w/v) sucrose into the tubes It is not essential that you keep the interfaces between the layers sharp, because the gradients are primarily being used for flotation during pelleting of the virus
11 Layer 10 mL of the extract over each of the sucrose gradients and centrifuge at 80,000*g* in a swinging bucket rotor for 3 h at 4°C
12 Remove the gradients and resuspend the first pellet in 5 mL of 10 m*M* potassium phosphate buffer, pH 6 8 Transfer the suspended pellet to the next tube and resuspend each pellet sequentially in the original 5 mL of buffer. Resuspend any clumps with a glass homogenizer immediately, because aggregation can occur at this point If the pellets are green, they should be diluted to 9 5 mL with 10 m*M* potassium phosphate buffer, pH 6 8, and 0 5 mL of 20% (w/v) Triton X-100, thoroughly mixed into the solution This is followed by repelleting through a single 6–24% (w/v) sucrose gradient, as described in **step 10**.
13 Immediately centrifuge the suspended pellets at 2000*g* for 10 min at 4°C to remove insoluble material
14 Remove the supernatant and measure the absorbance spectrum from 220 to 320 nm The extinction coefficient at 260 nm is 2 6 OD/mg for BSMV
15 Normal yields for BSMV from barley leaves are 1–2 mg virus/g tissue
16. If the preparation is to be stored, add ethylene glycol to 5% and store at –20°C (*see* **Note 2**)

### 3.2. Viral RNA Extraction

This protocol is based on a modification of the protocol described by Jackson and Brakke ***(15)***.

1 Dilute the virus to approx 5 mg/mL in 10 m*M* potassium phosphate buffer, pH 6 8, or $H_2O$ to yield a volume of approx 2.5 mL
2 Add 100 µL of 2 mg/mL proteinase K and incubate on ice for 30 min
3 Add 2 6 mL of ammonium carbonate buffer, pH 9 0, and mix well
4 Add 5 2 mL of phenol·chloroform and vortex well to emulsify
5 Centrifuge at 7000*g* for 5 min to separate the aqueous and organic phases
6. Remove the upper aqueous phase, being careful not to disturb the interface, and transfer to another tube
7 Repeat **steps 4–6**
8 To precipitate the RNA, measure the volume of the aqueous phase and add 1/20th volume of 3*M* sodium acetate, pH 4 8, 2 vol of ethanol, and place at –20°C for several hours

9 Centrifuge at 7000*g* at 4°C for 15 min, decant the ethanol, and drain the tube well
10 Resuspend the pellet in 1 mL $H_2O$ and add 3 mL of 4*M* sodium acetate, pH 6 0, and incubate on ice for 2 h. This step precipitates single-stranded RNA but tends to solubilize protein and DNA contaminants that are insoluble in ethanol
11. Centrifuge as before, drain the pellet well, and resuspend the pellet in 2 mL $H_2O$
**Note:** This pellet is more difficult to resuspend than the previous ones, so a larger volume of $H_2O$ is added Add 2 vol of ethanol, plus 0 05 vol of 3*M* sodium acetate, pH 4 8, incubate at –20°C, and centrifuge as before
12 Resuspend the pellet in 500 µL $H_2O$ and determine the concentration of RNA by measuring the absorbance at 260 nm and scan the sample from 210–310 nm 1 OD at 260 nm is equal to a concentration of 40 µg/mL RNA
13. To check that the RNA is not degraded, analyze a portion on a 1% (w/v) agarose gel prepared in 1X TAE

## 4. Notes

1 Hordeivirus particles tend to aggregate because the particles have a strong negative charge and, hence, polycations will interact with them When performing the virus purification, try to complete it in 1 d, because aggregation of the particles increases as the time used for purification increases
2 If BSMV is stored in 5% ethylene glycol at –20°C, it remains infectious for many years

## Acknowledgments

The financial support for this research was provided by the United States Department of Agriculture from 1978 to the present award, 94-37303-0766.

## References

1 Hamilton, R. I and Jackson, A O (1995) Hordeivirus, in *Virus Taxonomy, Sixth Report of the International Committee on Taxonomy of Viruses* (Murphy, F A , Fauquet, C M , Bishop D H L , Ghabrial, S A , Jarvis, A W , Martelli, G P , Mayo, M A , and Summers, M D , eds.), Springer-Verlag, Wien New York, pp 441–444
2 Gustafson, G and Armour, S L (1986) The complete sequence of RNA β from the type strain of barley stripe mosaic virus. *Nucleic Acids Res* **14,** 3829–3841
3. Gustafson, G , Hunter, B , Hanau, R , Armour, S L., and Jackson, A O. (1987) Nucleotide sequence and genetic organization of barley stripe mosaic virus RNA γ *Virology* **158,** 394–406
4 Gustafson, G , Armour, S L , Gamboa, G C , Burgett, S G , and Shepherd, J W (1989) Nucleotide sequence of barley stripe mosaic virus RNA α RNA α encodes a single polypeptide with homology to corresponding proteins from other viruses *Virology* **170,** 370–377
5 Petty, I T D , French, R , Jones, R W , and Jackson, A O (1990) Identification of barley stripe mosaic virus genes involved in viral RNA replication and movement *EMBO J* **9,** 1953–1957

6 Petty, I T. D and Jackson, A O (1990) Mutational analysis of barley stripe mosaic virus RNA β *Virology* **179,** 712–718
7 Donald, R G. K , Lawrence, D M , and Jackson, A O (1997) The barley stripe mosaic virus 58-kilodalton βb protein is a multifunctional RNA binding protein *J Virol* **71,** 1538–1546
8 Donald, R G K , Zhou, H , and Jackson, A O (1993) Serological analysis of barley stripe mosaic virus-encoded proteins in infected barley *Virology* **195,** 659–668
9 Zhou, H and Jackson, A O (1996) Expression of the barley stripe mosaic virus RNA β "triple gene block" *Virology* **216,** 367–379
10 Petty, I T D , French, R , Jones, R W , and Jackson, A O (1990) Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement *EMBO J* **9,** 3453–3457
11 Jackson, A O , Dawson, J R O , Covey, S N , Hull, R , and Davies, J W (1983) Sequence relations and coding properties of a subgenomic RNA isolated from barley stripe mosaic virus *Virology* **127,** 37–44
12. Donald, R G K and Jackson, A O (1996) RNA-binding activities of barley stripe mosaic virus αb fusion proteins *J Gen Virol* **77,** 879–888
13 Donald, R G K and Jackson, A O (1994) The barley stripe mosaic virus γb gene encodes a multifunctional cysteine-rich protein that affects pathogenesis *Plant Cell* **6,** 1593–1606
14 Fraenkel-Conrat, H , Singer, B , and Tsugita, A (1961) Purification of viral RNA by means of bentonite *Virology* **14,** 54–58
15 Jackson, A O and Brakke, M K (1973) Multicomponent properties of barley stripe mosaic virus ribonucleic acid *Virology* **55,** 483–494

# 9

# Furovirus Isolation and RNA Extraction

**Salah E. Bouzoubaa**

## 1. Introduction

The Furovirus (*fu*ngus-transmitted *ro*d-shaped) group, whose type member is soil-borne wheat mosaic virus (SBWMV) *(1)*, is a very heterogeneous group infecting a wide variety of hosts The group consists of viruses that are naturally transmitted by soil-borne fungal vectors, generally of the family Plasmodiophorales Virions are rigid rods and contain two or more genome components consisting of positive-sense RNA. Furoviruses typically infect roots, but can also invade, or be inoculated to, leaves. As sequence data for different furoviruses has become available, it has become clear that there are significant differences in genetic organization within the group *(2,10)*. We find it convenient to divide the viruses into two major subgroups (**Fig. 1**), which are distinguished by the nature of the protein(s) putatively involved in cell-to-cell movement. Subgroup 1 consists of SBWMV, which appears to have a movement protein (MP) of the type characterized by the 30-kDa protein of tobacco mosaic virus (TMV) *(6)* Subgroup 2 furoviruses (beet necrotic yellow vein virus [BNYVV], peanut clump virus [PCV], and potato moptop virus [PMTV]) do not have a TMV type MP but instead possess a cluster of three slightly overlapping genes known as the triple gene block (TGB), which has been shown for BNYVV *(11)*, and is presumed for the other viruses *(7–9)* to mediate viral cell-to-cell movement

Within Subgroup 2, there are further differences in genetic organization concerning the number of genome components (four for BNYVV, three for PMTV, and two for PCV) and the manner in which other genes are distributed upon them (**Fig. 1**). For example, in BNYVV and PCV, the TGB and the coat protein (CP) are situated on the same genome component; in PMTV they fall on separate RNAs. These changes have presumably arisen from gene shuffling in the

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

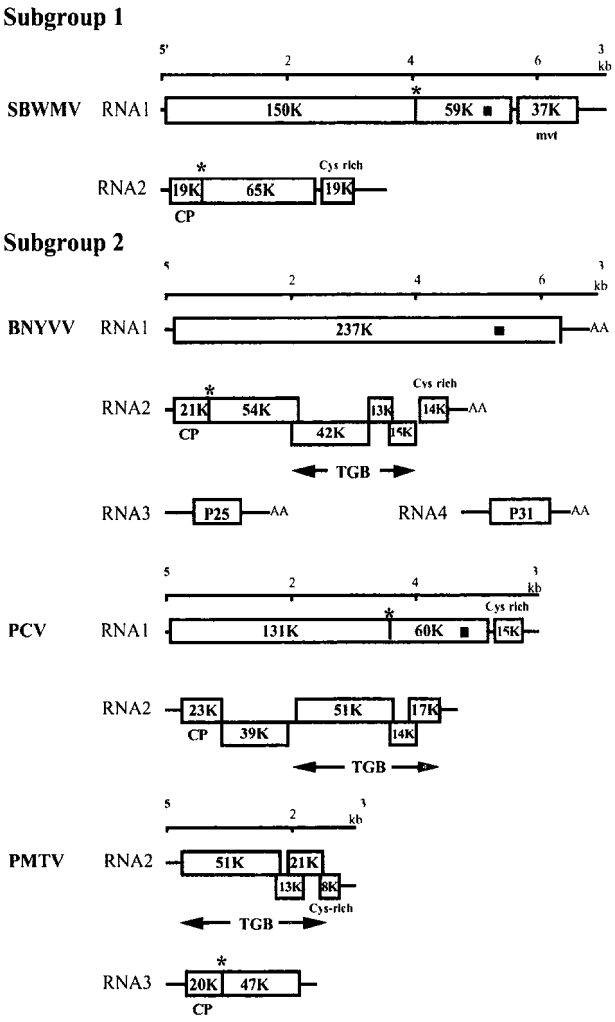



Fig 1 Genetic organization of characterized members of the furovirus group RNA1 of PMTV has not yet been sequenced, but presumably encodes the viral replicase ORFs are represented as hollow rectangles. Asterisks indicate suppressible termination codons TGB, tripe gene block, Cys-rich, cysteine rich protein, CP, coat protein, mvt, putative movement protein of SBWMV, AA, poly(A) tail, black rectangle, consensus core polymerase motif *(12)*.

course of evolution It should also be mentioned that the replicase sequence encoded by RNA1 of BNYVV is taxonomically distant from the putative replicases of the other furoviruses *(6,12)* In spite of the aforesaid differences, however, it is important to note several elements that are common to all four

sequenced furoviruses: first, the 5'-proximal position of the CP cistron on a genomic RNA; second, the presence of an open reading frame (ORF) adjacent to the CP cistron, which is expressed by translational readthrough for BNYVV *(13,14)*, SBWMV *(6,15)*, and probably PMTV *(10)*, and by ribosome scanning for PCV *(7,16)* In the case of BNYVV, this protein has been implicated in fungus transmission *(17)*. Finally, all the characterized furoviruses have an ORF for a small cysteine-rich protein, which has been shown in the case of BNYVV to be involved in regulation of CP expression *(18)*.

At present, a dozen viruses have been classified within the furovirus group *(19)*, and, for each of them, a distinct purification procedure (or several such procedures) has been described. Because of this diversity, this chapter will only detail the technique described by Putz and Kuszala (*see* **ref. *20)***, *which is* currently used in slightly modified form in this laboratory for large-scale purifications of BNYVV. However, significant differences with the purification methods for other well-studied furoviruses are mentioned in **Subheading 4**.

Generally speaking, furoviruses do not multiply well in their hosts *(19)*. Another obstacle to their purification is a tendancy of the virions to self-aggregate or to adhere to large cellular components. Aggregation is reported to be particularly severe in the case of PMTV and *Nicotiana velutina* mosaic virus (NVMV) *(21–23)*. Consequently, yields of purified virus rarely exceed 30 mg/kg of leaves However, the method described in this chapter has given yields of BNYVV of as much as 80 mg/kg of leaves of the local lesion host *Chenopodium quinoa*.

## 2. Materials

1 Plant host *C quinoa*, aged 7–8 wk
2 Inoculum. Freshly ground leaves of infected *C quinoa* Leaves that have been frozen for some weeks or lyophilized (preferable) can also be used
3 Inoculum buffer (4X): 0 2*M* potassium phosphate, pH 7 0
4 Abrasive powder Celite.
5. Sterile cotton, mortar, and plastic gloves
6. Variable speed food blender of approx 1-L vol
7. Extraction buffer. Sterile 0 1*M* sodium tetraborate, adjusted to pH 9 0 with boric acid
8. Clarification agent carbon tetrachloride ($CCl_4$)
9 Miracloth or equivalent filter tissue
10 Precipitation agent NaCl (8 g/L of clarified sap), polyethylene glycol (PEG) 6000 (20 g/L of clarified sap)
11. 20% Sucrose cushion (w/v) prepared in 10 m*M* extraction buffer
12 Low-speed centrifuge equipped with fixed-angle rotors and with tubes of capacity approx 0.5 L, 0 25 L, and 30 mL Ultracentrifuge with fixed-angle rotor and accepting ultracentrifuge tubes of approx 25 mL

13 RNA extraction. Phenol saturated with 10 m*M* Tris-HCl pH 8 0 Saturated phenol chloroform (1 1). Distillated ethanol Sterile 5*M* NaCl Sterile $dH_2O$ Microcentrifuge tubes

## 3. Method

### 3.1. Virus Isolation

1 For the inoculum, grind heavily infected *C quinoa* leaves in a cold mortar in 50 m*M* inoculum buffer. About 30 mL of buffer is used for five leaves. This amount of inoculum is sufficient for inoculation of 60 *C quinoa* plants, which will yield approx 200 g of infected leaves (*see* **Note 1**) for virus extraction Be careful to maintain the inoculum on ice at all times. Leaves sprinkled with Celite are mechanically inoculated with a wad of cotton saturated with inoculum When all the plants are inoculated, wash the leaves with water in order to eliminate excess inoculum and Celite

2. After inoculation, the plants are maintained for 9–13 d in the greenhouse (*see* **Note 2**) in the following conditions 16 h light at 22–24°C, 8 h dark at 16–18°C, with 65–85% humidity. Infected leaves are harvested, weighed, and homogenized in the blender in the presence of 170 mL cold extraction buffer and 100 mL cold $CCl_4$ for 100 g of leaves (*see* **Note 3**). Add the leaves little by little at low speed, and, when all the leaves are in the blender, cover it and blend at high speed for 2–3 min to obtain a uniform homogenate

3 Decant the homogenate into the 0 5-L centrifuge tubes and centrifuge at 9000*g* for 30 min at 4°C.

4 Filter the supernatant through Miracloth into a big beaker or other recipient Measure the volume and then add (with stirring) solid NaCl, and then solid PEG, to concentrations of 0 8 and 2%, respectively

5 Let the mixture precipitate for at least 2 h in the cold room, with gentle stirring

6 Pour the liquid into the 0 25-L centrifuge tubes and centrifuge at 17,000*g* for 20 min at 4°C

7 Resuspend the pellets in 100 mL (total volume) of 10 m*M* extraction buffer and let the virus resuspend overnight at 4°C, with gentle stirring The following day, centrifuge the viral suspension in 30-mL centrifuge tubes at 5000*g* for 20 min (*see* **Note 4**) to remove debris

8 Combine the supernatants and repeat the precipitation (0 8% NaCl, 2% PEG) for 2 h at 4°C

9 Centrifuge the viral suspension in the 30-mL centrifuge tubes at 5000*g* for 30 min The pellets are resuspended in 35 mL (total volume) of 10 m*M* extraction buffer overnight, with gentle stirring at 4°C

10 The viral suspension is again clarified by 30 min centrifugation at 5000*g* at 4°C and the supernatant is retained

11. Two 25-mL ultracentrifuge tubes are each filled with 7 mL of a 20% sucrose cushion, upon which 17.5 mL of the supernatant is overlaid The tubes are centrifuged for 2 h at 100,000*g* at 4°C

12 The viral pellets are gently resuspended overnight in 0 5 mL of 10 m*M* extraction buffer and the optical density (OD) at 260 nm is measured on an aliquot after a 1 100 dilution The virus concentration (C) is established according to the following formula C (mg/mL) = OD × 100/3 2 The number in the denominator is the weight extinction coefficient ***(20)*** In general, we obtain, depending on the viral isolate, 20–80 mg/kg of leaves (*see* **Note 5**). The virus so obtained can be stored at 4°C for a few days or frozen at –20°C for several months, but its infectivity decreases abruptly after the first few days.

### 3.2. RNA Extraction

1 For RNA extraction, transfer the viral suspension to Eppendorf tubes and add NaCl to a final concentration of 200 m*M* Extract with phenol for 5 min, with the tubes being placed in ice from time to time Mix vigorously during the phenol extraction, to produce a good emulsion Centrifuge for 5 min at 8000*g* and remove the supernatant to new sterile tubes Repeat the extraction two times, but with phenol chloroform rather than phenol Add 2 vol of ethanol to the supernatant and let the RNA precipitate at –20°C The RNA can be stored in this form for several months or years, and then collected by centrifugation at 17,000*g* for 15 min. The RNA pellet is washed once with 70% ethanol, followed by centrifugation, as above, the pellet is dried and then dissolved in a small volume of sterile water

2 The RNA concentration can be determined by spectrophotometry at 260 nm The concentration (mg/mL) = OD × dilution factor/25 The viral RNA constitutes approx 5% of the particle, with an 80% RNA extraction yield, we obtain 0 8–4 mg of viral RNA from a virus preparation

## 4. Notes

1 Note that the host used is *C quinoa,* which gives a local lesion response to BNYVV The lesions differ somewhat in size and appearance, depending on the viral isolate Isolates carrying RNA3 produce intense yellow lesions, which have a tendency to extend along the veins ***(24)*** These isolates give the best purification yield Isolates carrying only RNA-1 and RNA-2 induce mild chlorotic local lesions and give a lower purification yield. Systemic infection hosts, such as spinach (*Spinacia oleracea*), did not give superior yields of BNYVV ***(20)***. For other furoviruses (for example, SBWMV or PMTV ***(23,25,26)***, systemic infection hosts have been used for viral propagation and purification

2 The viral multiplication period depends on the season, being short in the summer, when the light intensity is strong, and longer in the winter The best time to harvest infected leaves is when the lesions are well visible, but the leaves are still green Do not wait until the leaves become senescent For other furoviruses (SBWMV or PMTV), leaves are harvested for 3–4 wk postinoculation ***(25,26)***

3 The clarification agent changes from one virus to another For PCV, the organic solvent is a mixture of butanol and chloroform ***(27)*** Sap clarification with an organic solvent is omitted for viruses such as PMTV or NVMV ***(21–23)***, for

which heavy losses caused by aggregation would occur during centrifugation These viruses are purified from pellets obtained during the first sap centrifugation, using Triton X-100 and/or urea as detergent

4 Large cellular components are eliminated at this stage, but there is a risk of also losing aggregated viral particles So it is necessary to let the virus resuspend slowly at 4°C with gentle stirring Some viral particles may be damaged, but the losses will be less important during centrifugation (Ethylenedinitrolo)tetra-acetic acid (EDTA), which is employed in many virus purification protocols, and which is thought to favor virus disaggregation, is, curiously, not used in most furovirus purification protocols

5. The virus can be further purified by ultracentrifugation through a Nycodenz (Nycomed, Oslo) gradient ***(28)*** In this case, the virus is adjusted to a volume of 8 4 mL, with 10-times-diluted extraction buffer, and 5.85 g of Nycodenz are added The volume is then adjusted to 11 5 mL with the same buffer, followed by centrifugation at 300,000*g* overnight at 15°C in a vertical rotor The opalescent band is collected and diluted with the 10-times-diluted extraction buffer Virus is then concentrated by ultracentrifugation

## References


1 Brunt, A A (1991) Soil-borne wheat mosaic virus group, in *Classification and Nomenclature of Viruses Fifth Report of the International Commitee on Taxonomy of Viruses* (Francki, R I B , Fauquet, C M., Knudson, D L , and Brown, F , eds ), Springer-Verlag, New York, pp 377–379

2 Bouzoubaa, S., Guilley, H , Jonard, G., Richards, K E , and Putz, C (1985) Nucleotide sequence analysis of RNA-3 and -4 of beet necrotic yellow vein virus, isolates F2 and G1 *J Gen Virol* **66,** 1553–1564

3. Bouzoubaa, S , Ziegler, V , Beck, D., Guilley, H , Richards, K. E and Jonard, G (1986) Nucleotide sequence of beet necrotic yellow vein virus RNA-2 *J Gen Virol* **67,** 1689–1700

4 Bouzoubaa, S , Quillet, L Guilley, H , Jonard, G , and Richards, K E , (1987) Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *J Gen Virol* **68,** 615–626.

5 Richards, K. E and Tamada, T (1992) Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annu Rev Phytopath* **30,** 291–313

6 Shirako, Y and Wilson, T M A. (1993) Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus *Virology* **195,** 16–32

7. Manohar, S. K , Guilley, H., Dollet, M., Richards, K , and Jonard, G (1993) Nucleotide sequence and genetic organization of peanut clump virus RNA 2 and partial characterization of deleted forms *Virology* **195,** 33–41

8 Herzog, E., Guilley, H., Manohar, S K , Dollet, M., Richards, K., Fritsch, C , and Jonard, G (1994) Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses *J Gen Virol* **75,** 3147–3155

9 Scott, K P, Kashiwazaki, S, Reavy, B, and Harrison, B D (1994) The nucleotide sequence of potato mop-top virus RNA 2 a novel type of genome organization for a furovirus *J Gen Virol* **75,** 3561–3568
10. Kashiwazaki, S., Scott, K. P, Reavy, B, and Harrison, B D. (1995) Sequence analysis and gene content of potato mop-top virus RNA-3 Further evidence of heterogeneity in the genome organization of furoviruses *Virology* **206,** 701–706
11. Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H, Richards, K E., and Jonard, G. (1992) Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA-2 *Virology* **189,** 40–47
12. Koonin, E. V (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses *J Gen Virol* **72,** 2197–2206
13 Ziegler, V, Richards, K E, Guilley, H., Jonard, G, and Putz, C (1985) Cell-free translation of beet necrotic yellow vein virus Readthrough of the coat protein cistron *J Gen Virol* **66,** 2079–2087
14 Schmitt, C, Balmori, E, Jonard G, Richards, K E, and Guilley, H (1992) *In vitro* mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA-2. evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly *Proc Natl Acad Sci USA* **89,** 5715–5719
15 Shirako, Y. and Ehara, Y (1986) Comparison of the *in vitro* translation products of wild-type and a deletion mutant of soil-borne wheat mosaic virus *Virology* **195,** 16–32
16 Herzog, E, Guilley, H, and Fritsch, C (1995) Translation of the second gene of peanut clump virus RNA-2 occurs by leaky scanning *in vitro Virology* **208,** 215–225
17. Tamada, T and Kusume, T (1991) Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus *Polymyxa betae J Gen Virol* **72,** 1497–1504
18 Hehn, A, Bouzoubaa, S, Marbach, J., Bate, N, Twell, D, Richards, K E, Guilley, H, and Jonard, G (1995) The small cysteine-rich protein P14 of beet necrotic yellow vein virus regulates accumulation of RNA 2 in *cis* and coat protein in *trans Virology* **210,** 73–81
19 Brunt, A A and Richards, K. E (1989). Biology and molecular biology of furoviruses *Adv Virus Res* **36,** 1–32
20 Putz, C. and Kuszala, M (1978) La rhizomanie de la betterave sucrière en Alsace Recherche d'une nouvelle méthode de purification du "Beet Necrotic Yellow Vein Virus" *Ann Phytopathol* **10,** 247–262
21 Harrison, B D and Jones, R A. C (1970) Host range and some properties of potato mop-top virus. *Ann Appl Biol* **65,** 393–402
22. Kassanis, B., Woods, R D, and White, R F (1972) Some properties of potato mop-top virus and its serological relationship to tobbaco mosaic virus *J Gen Virol* **14,** 123–132
23 Randles, J W, Harrison, B D, and Roberts, M (1976) *Nicotiana velutina* mosaic virus purification, properties and affinities with other rod-shaped viruses *Ann Appl Biol* **84,** 193–204

24 Jupin, I , Guilley, H , Richards, K. E., and Jonard, G. (1992) Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves *EMBO J* **11,** 479–488

25 Rao, A S and Brakke, M K (1970) Dark treatment of wheat inoculated with soil-borne wheat mosaic and barley stripe mosaic viruses *Phytopathology* **60,** 714–716

26 Torrance, L , Cowan, G H , and Pereira, L G (1993) Monoclonal antibodies specific for potato mop-top virus, and some properties of the coat protein *Ann Appl Biol* **122,** 311–322

27 Reddy, D V R , Robinson, D J , Roberts, I M , and Harrison, B D (1985) Genome properties and relationships of Indian Peanut Clump Virus *J Gen Virol* **66,** 2011–2016

28 Gugerli, P (1984) Isopicnic centrifugation of plant viruses in Nycodenz density gradients. *J Virol Methods* **9,** 249–258.

# 10

# Tobravirus Isolation and RNA Extraction

**Sally C. Taylor**

## 1. Introduction

The tobraviruses have a genome consisting of two positive-sense, single-stranded RNA molecules The two genomic RNAs, RNA-1 and RNA-2, are encapsidated separately in rod-shaped particles with lengths of 180–215 nm (L particles) and 46–115 nm (S particles), respectively (**Fig. 1**) *(1,2)*. Both the RNAs are encapsidated by a single species of coat protein (CP) with a mol wt of approx 23 kDa. Tobraviruses can be divided into three serologically distinct subgroups· tobacco rattle virus (TRV), which is the type member, pea early browning virus (PEBV), and pepper ringspot virus (PRV). In the field, these viruses are transmitted by soil-inhabiting nematodes of the family *Trichodoridae*, and certain isolates are also transmitted through the seed. Tobraviruses can infect a wide range of plant species including economically important crops such as potatoes and ornamental bulbs.

The nucleotide sequence of several tobraviruses has now been determined *(3–8)*. This revealed that RNA-1 of each of the three virus subgroups is of a similar size (6.8–7.0 kb) and contains at least four open reading frames (ORFs) (**Fig. 1ai** and **bi**). RNA-2, however, varies considerably in length (1 8–4.5 kb), even between isolates of the same subgroup, and may contain 1–4 ORFs (**Fig. 1aii** and **bii**). Both RNA-1 and RNA-2 have a 7-methylguanosine cap structure at the 5' end and a tRNA-like structure at the 3' end *(2,9)*.

The genomic organization of RNA-1 of tobraviruses is very similar to the monopartite tobamovirus, tobacco mosaic virus (TMV) *(10)*. The two 5' proximal ORFs on RNA-1 of the type member TRV encode proteins of 134 and 194 kDa. The 134-kDa protein terminates in an opal (UGA) stop codon and readthrough of this codon results in the production of a 194-kDa protein. The 134- and 194-kDa proteins have been found to share amino acid homology

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

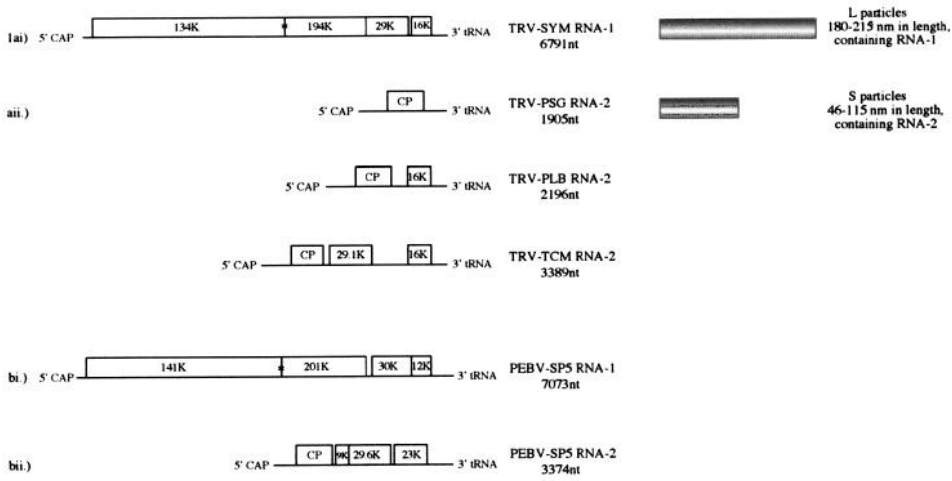



Fig. 1. Tobravirus structure and genome organization. (ai) Genome organization of RNA-1 of TRV isolate SYM. (aii) Genome organization of RNA-2 of various TRV isolates. (bi and bii) Genome organization of RNA-1 and RNA-2 of PEBV isolate SP5. RNA is shown as a horizontal line and the positions of the open reading frames (ORFs) are indicated by boxes. The numbers inside the boxes are the approximate mol wt in kDa of the proteins encoded by each ORF. The coat protein ORF is denoted by CP. The position of the readthrough termination codon on RNA-1 is indicated by an asterisk.

with the TMV 126- and 183-kDa replicase proteins. The third ORF on TRV RNA-1 encodes a 29-kDa protein that has homology with the TMV 30-kDa movement protein. Unlike TMV, the 3' proximal ORF on TRV RNA-1 does not encode the CP, but encodes a cysteine-rich 16-kDa protein. Although the function of this protein is not yet known, it is thought that in PEBV the equivalent 12-kDa protein may have a role in virus multiplication (S. A. MacFarlane, personal communication).

The TRV 29- and 16-kDa proteins are coded by RNA-1, but they are not translated directly from the genomic RNA and are expressed from individual subgenomic RNA species that are 3' coterminal with RNA-1 ***(11)***. TRV RNA-1 also has the potential to code for a 59-kDa protein by translation of an internal ORF. This ORF initiates at an AUG codon present 87 nucleotides downstream of the termination codon for the 134-kDa protein and terminates at the stop codon for the 194-kDa protein. This protein has not been detected; however, plants transformed with the equivalent readthrough regions of PEBV and TMV have been found to be resistant to subsequent infections with the same viruses ***(12,13)***.

The tobraviral CP is the 5' proximal gene on RNA-2, but it is not translated efficiently from the genomic RNA and is expressed from a 3' coterminal subgenomic RNA *(4,5)*. The CP is thought to be an important determinant of nematode transmission *(14)*. Additional ORFs have been identified on RNA-2 of several tobravirus isolates. Some TRV RNA-2 species have undergone recombination in the 3' region with part of the same region of RNA-1, and thus carry part or all of the RNA-1-coded 29K and 16K genes *(3–5,15)* TRV isolate TCM and PEBV isolate SP5, RNA-2, contain an ORF for a 29.1- and a 29.6-kDa protein, respectively *(5,8)*. Sequence comparison of RNA-2 of nematode-transmittable and nematode-nontransmittable isolates of PEBV suggest that the 29 6-kDa protein may be involved in nematode transmission *(16)*. There is evidence that the additional 9- and 23-kDa ORFs on PEBV (SP5) RNA-2 may also have some function in vector transmission (S. A MacFarlane, personal communication).

## 2. Materials

### 2.1. Purification of Virus

1 Celite
2. 30 m*M* Sodium phosphate buffer, pH 7 5
3 Virus extraction buffer 50 m*M* sodium phosphate buffer, 0 15% thioglycollic acid (v/v) Add 1 5 mL of thioglycollic acid to 250 mL 200 m*M* $Na_2HPO_4$ and 550 mL of distilled $H_2O$, adjust to pH 7 5 with NaOH, and make up to 1 L with distilled $H_2O$ Make buffer up fresh and keep at 4°C (**Caution:** Wear gloves when handling thioglycollic acid and use in a fume hood )
4 Commercial blender
5 Muslin
6 Polyethelene glycol (PEG), mol wt = 6000
7 Sodium chloride (NaCl)
8 Chloroform·butan-1-ol 1 1 (v/v) (**Caution:** Wear gloves and use solvents in a fume hood.)
9 Sucrose cushion. 30% sucrose in 30 m*M* sodium phosphate buffer, pH 7 5
10 10 m*M* Tris-HCl, pH 7 5

### 2.2. Extraction of Viral RNA

1 Deoxyribonuclease I (DNase I) RNase-free
2 1X DNase reaction buffer· 50 m*M* Tris-HCl, pH 7 9, 5 m*M* $MgCl_2$
3 EDTA
4 Proteinase K
5 1X Proteinase K buffer 100 m*M* Tris-HCl, pH 7 4, 1 m*M* EDTA, 0 1% SDS
6 Tris-equilibrated phenol, pH 8 0 (**Caution:** Wear gloves and use in fume hood)
7 Chloroform/isoamyl alcohol 24 1 (**Caution:** Wear gloves and use in fume hood)
8 Phenol/chloroform isoamyl alcohol 1 1

9 3*M* Sodium acetate, pH 5 5
10. 70 and 100% Ethanol

## 3. Methods

### 3.1. Purification of Virus

The virus purification method described in this chapter has been developed to purify PEBV from *Nicotiana benthamiana* or *Nicotiana clevelandii* This method has proved equally successful for purifying TRV from *Nicotiana tabacum*. An alternative method is also included (*see* **Note 4**). *Chenopodium amaranticolor* is a local lesion host for both viruses and can therefore be used as an indicator plant for infection

1 Take plants at the 4–5 leaf stage, dust one leaf with Celite and mechanically inoculate with virus (*see* **Notes 1** and **2**)
2. Harvest plants 10–12 d postinoculation and, using a blender, homogenize the tissue in approx 2 vol of 50 m*M* sodium phosphate buffer, pH 7 5, containing 0 15% (v/v) thioglycollic acid, adjusted to pH 7 5, with NaOH (*see* **Notes 3** and **4**)
3. Freeze the homogenate at –20°C for 1–2 wk, and then allow it to thaw slowly overnight to room temperature
4 Clarify the homogenate by filtering through two layers of muslin, followed by centrifugation at 10,000*g* for 5 min at 4°C
5 To precipitate virus particles from the supernatant, add PEG 6000 to a final concentration of 10% (w/v) and NaCl to 2% (w/v), at room temperature, then stir slowly at 4°C for 3 h (*see* **Note 5**)
6 Pellet the virus by centrifugation at 15,000*g* for 15 min at 4°C, and resuspend in 30 m*M* sodium phosphate buffer, pH 7 5 (*see* **Note 6**)
7 Clarify the resuspended virus by centrifugation at 10,000*g* for 5 min
8 To remove any remaining plant material, extract the virus suspension with an equal volume of chloroform butan-1-ol 1 1 Collect the upper layer containing the virus by centrifugation at 2500*g* for 5 min
9 Re-extract the chloroform butanol lower layer with distilled water, then pool the aqueous upper layers and re-extract with chloroform·butanol (*see* **Note 7**)
10 Pellet the virus by centrifugation for 4 h at 85,000*g* and 18°C and resuspend in 10 m*M* Tris-HCl, pH 7 5 (*see* **Note 8**)
11 If the virus is to be further purified, resuspend the viral pellet in 30 m*M* sodium phosphate buffer, pH 7 5, and layer the suspension onto a 30% sucrose cushion Sediment the virus by centrifugation for 2–3 h at 140,000*g* and 15°C in a swing-bucket rotor and resuspend the pellet in 10 m*M* Tris-HCl, pH 7 5 (*see* **Note 9**)
12 To determine the concentration of the virus measure the optical density (OD) at 260 nm. An $OD_{260}$ of 3 = 1 mg/mL for TRV and PEBV. An $OD_{260}/OD_{280}$ ratio of 1 15 is expected for a pure tobravirus prep
13. Store virus at 4°C

### *3.2. Extraction of viral RNA*

1. To remove any contaminating host DNA, incubate the purified virus for 2 h on ice with 25 µg/mL RNase-free DNase I in 1X DNase buffer Stop the reaction by adding EDTA to 17 m*M*
2. Incubate the virus at 37°C for 2 h with 200 µg/mL proteinase K in 1X proteinase K buffer to isolate RNA from the virus particles (*see* **Note 10**)
3. To extract the RNA, add 1 vol of phenol, and vortex vigorously Separate the two phases by centrifugation at 10,000*g* for 5 min
4. Remove the aqueous upper layer and re-extract it with an equal volume of phenol chloroform-isoamylalcohol (1 1)
5 Pool the aqueous layers and precipitate the RNA with 0 1 vol of 3*M* sodium acetate, pH 5 5, and 2 5 vol of ethanol and collect the RNA by centrifugation
6. Wash the pellet with 70% ethanol, then dry the RNA under vacuum and resuspend in sterile distilled water
7 To determine the concentration of RNA measure the OD at 260 nm using an $OD_{260}$ of 25 = 1 mg/mL, or, alternatively, estimate the concentration after analysis by electrophoresis on an denaturing agarose gel (*see* **Note 11**)

## 4. Notes

1 Sap from a virus-infected plant or purified virus can be used as an inoculum Sap is prepared by grinding virus-infected tissue in 30 m*M* sodium phosphate buffer, pH 7 5 (1 g tissue/1 mL buffer) The sap is then diluted 1 10 with 30 m*M* sodium phosphate buffer, pH 7 5, and 20 µL is inoculated onto each plant The inoculum is stored at –20°C in an undiluted form Alternatively, plants can be inoculated with purified virus at a concentration of approx 10 µg/mL
2. Local lesions should appear on *Chenopodium* indicator plants 3–5 d postinoculation, confirming the infectivity of the inoculum
3 Harvest whole plants, remove the roots, and homogenize the remaining plant material 200–300 g of plant tissue should yield approx 20–30 mg of virus
4 Tobraviruses can be purified using an alternative method that avoids the use of thioglycollic acid and solvents, and instead involves repeated cycles of low- and high-speed centrifugation. Omit thioglycollic acid from the extraction buffer and homogenate the tissue in 50 m*M* sodium phosphate buffer. pH 7.5 Freeze the homogenate at –20°C for approx 2 wk, thaw overnight, and then proceed with **steps 4–7** of **Subheading 3.1.** Obmit the solvent extraction **steps 8** and **9** of **Subheading 3.1.** and proceed with **step 10** of **Subheading 3.1.** Repeat the low- and high-speed centrifugations (**Subheading 3.1., steps 7** and **10**) until all traces of pigment and other contaminating materials are removed and the viral pellet is white The purity of the virus prep can be assessed by determining the $OD_{260}/OD_{280}$ ratio A ratio of 1 15 is expected for a pure tobravirus prep
5. The solution should be stirred at room temperature until all the PEG dissolves, and then stirred in a cold room for 3 h

6 Resuspend virus pellet in approx 40 mL of 30 m*M* sodium phosphate buffer, pH 7 5, clarify as in **Subheading 3.1., step 7**, and then split into two tubes before extracting with chloroform butanol
7 Chloroform butanol extractions should be repeated until no contaminants are visible at the interface
8 The purification procedure can be stopped at **step 10** of **Subheading 3.1.**, and the viral pellet resuspended in 20–30 mL 10 m*M* Tris-HCl, pH 7 5 This should result in a virus preparation at approx 1 mg/mL, from an initial 200–300 g of infected tissue. To determine the exact virus concentration, measure the OD at 260 nm (*see* **Subheading 3.1., step 12**) If a purer virus preparation is required, then the viral pellet should be resuspended in 20–30 mL 30 m*M* sodium phosphate buffer, pH 7.5, and centrifuged through a sucrose pad to remove any remaining contaminants
9 Carefully layer 7 mL of virus onto 3 mL of 30% sucrose in 30 m*M* sodium phosphate buffer, pH 7 5, and sedimented by centrifugation for 2–3 h at 140,000*g* and 15°C in a swing-bucket rotor
10. The virus suspension should clear after incubation with proteinase K
11 TRV RNA should be visible as two bands of approx 6 8 kb and 1 8–3 9 kb, and PEBV as two bands of approx 7 0 and 3 4 kb on denaturing agarose gels. Additional smaller bands may also be visible These are subgenomic RNA species

## References

1 Murphy, F A , Fauquet, C M , Elishop, D H L , Ghabrial, S A , Jarvis, A W , Martelli, G P , Mayo, M A , and Summers, M D , eds (1995) *Classification and Nomenclature of Viruses Sixth Report of the International Commitee on Taxonomy of Viruses* Springer-Verlag, Wein and New York, pp 438–440.
2 Harrison, B D and Robinson, D J (1986) Tobraviruses, in *The Plant Viruses*, vol 2 (van Regenmortel, M H V, and Fraenkel-Conrat, H , eds ), Plenum, New York, pp 339–369
3 Bergh, S T , Koziel, M G , Huang, S C , Thomas, R A , Gilley, G P , and Seigel, A (1985) The nucleotide sequence of tobacco rattle virus RNA-2 (CAM strain) *Nucleic Acids Res* **13,** 8507–8518
4 Cornelissen, B J C , Linthorst, H J M , Brederode, F T , and Bol, J F (1986) Analysis of the genome structure of tobacco rattle virus strain PSG *Nucleic Acids Res* **14,** 2157–2169.
5 Angenent, G C , Linthorst, H J M , van Belkum, A F , Cornelissen, B J C , and Bol, J. F (1986) RNA-2 of tobacco rattle virus strain TCM encodes an unexpected gene *Nucleic Acids Res* **14,** 4673–4682
6 Hamilton, W D O , Boccara, M , Robinson, D J , and Baulcombe, D C (1987) The complete nucleotide sequence of tobacco rattle virus RNA-1 *J Gen Virol* **68,** 2563–2575
7 MacFarlane, S. A , Taylor, S. C , King, D I., Hughes, G., and Davies, J. W. (1989) Pea early browning virus RNA-1 encodes four polpeptides including a putative zinc-finger protein *Nucleic Acids Res* **17,** 2245–2260

8 Goulden, M G , Lomonossoff, G P, Davies, J W , and Wood, K R. (1990) The complete nucleotide sequence of PEBV RNA-2 reveals the presence of a novel open reading frame and provides insights into the structure of tobraviral subgenomic promoters *Nucleic Acids Res* **18,** 4507-4512

9 van Belkum, A , Cornelissen, B , Linthorst, H , Bol, J , Pley, C , and Bosch, L (1987) tRNA-like properties of tobacco rattle virus RNA *Nucleic Acids Res* **15,** 2837–2850

10 Goelet, P., Lomonossoff, G P., Butler, P J. G., Akam, M E., Gait, M J., and Karn, J (1982) Nucleotide sequence of tobacco mosaic virus RNA *Proc Natl Acad Sci. USA* **79,** 5818–5822

11 Boccara, M , Hamilton, W D O , and Baulcombe, D C (1986) The organisation and interviral homologies of genes at the 3' end of tobacco rattle virus RNA-1 *EMBO J* **5,** 223–229

12. Golemboski, D B., Lomonossoff, G. P , and Zaitlin, M. (1990) Plants transformed with a tobacco mosaic virus non-structural gene sequence are resistant to the virus *Proc Natl Acad Sci USA* **87,** 6311–6315.

13. MacFarlane, S. A and Davies, J W (1992) Plants transformed with a region of the 201-kilodalton replicase gene from pea early browning virus RNA-1 are resistant to virus infection. *Proc Natl Acad Sci USA* **89,** 5829–5833

14 Ploeg, A T , Robinson, D J , and Elrown, D J F (1993) RNA-2 of TRV encodes the determinants of transmissibility by trichodorid vector nematodes *J Gen Virol* **74,** 1463–1466

15 Angenent, G C , Posthumus, E , Brederode, F. T , and Bol, J F (1989) Genome structure of tobacco rattle virus strain PLB further evidence on the occurence of RNA recombination among tobraviruses *Virology* **171,** 271–274

16 MacFarlane, S A and Brown, D J F (1995) Sequence comparison of RNA-2 of nematode-transmissible and nematode-non-transmissible isolates of pea early browning virus suggest that the gene encoding the 29 kDa proetin may be involved in nematode transmission *J Gen Virol* **76,** 1299–1304

# 11

# Tobamovirus Isolation and RNA Extraction

**Sean N. Chapman**

## 1. Introduction

The tobamoviruses produce rigid, rod-shaped particles, with dimensions of approx 300 × 18 nm, and form one of the most extensively studied groups of plant viruses *(1)*. Members of the group infect a wide range of angiosperms, and individual members frequently have wide experimental host ranges. Tobamoviruses cause diseases in tobacco, tomato, pepper, orchid, cucumber, melon, bean, and crucifer plants. The characteristic symptoms of disease are stunting and chlorotic mosaics, mottles, or ringspots. These symptoms are often accompanied by distortion of leaves and fruits.

The tobamoviruses form particles that contain a single-stranded genomic RNA. Complete genomic RNAs of the majority of the definitive tobamoviruses have been cloned and their nucleotide sequences determined. The genomic organization of each of these viruses is very similar and each have four open reading frames (ORFs) The best characterized of the tobamoviruses is the type member tobacco mosaic virus (TMV) (**Fig. 1**) The TMV genomic RNA has a 7-methylguanosine cap structure at the 5' end *(2)*, followed by an untranslated leader sequence of 68 nucleotides, which is an enhancer of translation *(3)*. Downstream of the untranslated region are two ORFs that encode proteins of 126 and 183 kDa *(4)*, which are involved in the viral replication process *(5)*. Translation of the two proteins, which occurs from the genomic RNA, is initiated at the same methionine codon. The larger 183-kDa protein is produced by readthrough of a leaky amber stop codon at the end of the 126-kDa ORF, with a frequency of about 5% *(6)*. The third ORF overlaps the 183-kDa ORF by 17 nucleotides, but is out of frame. The ORF encodes a protein of 30-kDa that is produced by translation of a subgenomic RNA, which is 3' coterminal with the genomic RNA. The 30-kDa protein is necessary for intercellular movement of

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

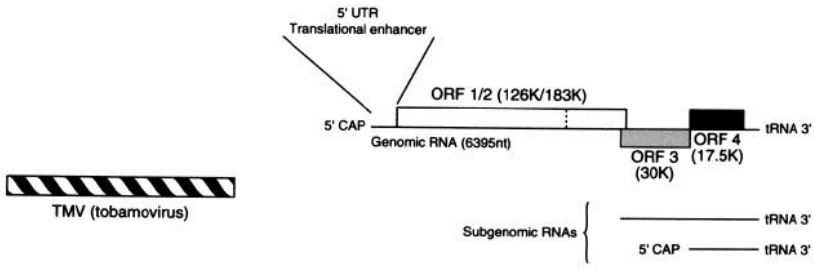



Fig. 1. Tobacco mosaic virus structure and genome organization. The positions of ORFs in the genomic RNA are indicated by boxes. The sizes of the proteins in kDa produced from these ORFs are indicated in brackets. The 17.5-kDa CP is encoded by ORF 4. Additional features of the genomic and subgenomic RNAs are indicated above and below.

the virus *(7)*. The protein has been shown to increase the plasmodesmatal size exclusion limit *(8)* and to have nucleic acid-binding activity *(9)*. The fourth ORF encodes the coat protein (CP), which is required for virion formation and systemic movement of the virus *(10)*. The CP is produced from a second 3' coterminal subgenomic RNA that is capped at its 5' end, and starts nine nucleotides upstream of the initiating methionine of the CP. The origin of assembly site (OAS) *(11)*, from which particle formation is initiated, is located within the third ORF. Some other tobamoviruses have an OAS in the CP gene. Hence, depending on the tobamovirus, one or both subgenomic RNAs can be encapsidated. The TMV CP gene is followed by an untranslated region that contains several pseudoknot sequences, which are involved in translational enhancement *(12)*. At the 3' end of the genomic RNA is a tRNA-like structure that can be aminoacylated.

TMV was the first virus to be purified, in 1935 *(13)*, and since then many methods have been developed for its purification. Tobamoviruses have proven easy to purify because of the high accumulation of viral particles in many host plants, and because the particles are stable under a wide range of chemical and physical conditions. The purification method described in **Subheading 3.1.** has been chosen because of its simplicity, and because it does not involve ultracentrifugation. The method presented is based on that described by Gooding and Hebert *(14)*. Purification is dependent on the process of virion precipitation in the presence of the hydrophilic polymer polyethylene glycol (PEG), described by Leberman *(15)*.

## 2. Materials

1 0.5*M* Phosphate buffer Prepare a 0 5*M* solution of disodium hydrogen orthophosphate and adjust the pH to 7.2 with 0.5*M* potassium dihydrogen orthophosphate
2 Virion extraction buffer. Add 1% (v/v) 2-mercaptoethanol to 0.5*M* phosphate buffer just before use (*see* **Note 1**)
3 Acid-washed sand
4 Miracloth· purchased from Calbiochem (San Diego, CA) (*see* **Note 2**)
5 Butan-1-ol
6 20% (w/v) PEG (average mol wt 8000)
7 10 m*M* phosphate buffer. Prepared by 50-fold dilution of 0.5*M* phosphate buffer described above
8. 5*M* Sodium chloride
9. 5X RNA extraction buffer 0 5*M* sodium chloride, 5 m*M* ethylenediaminetetra-acetic acid disodium salt, 5% (w/v) sodium dodecyl sulfate, 0.1*M* Tris(hydroxymethyl)-methylamine (Tris), pH adjusted to 8 0 with hydrochloric acid
10 Phenol chloroform Dissolve 250 g of molecular biology grade phenol in 250 mL of 0 1*M* Tris-Cl, pH 8 0, and add 1 25 g of 8-hydroxyquinoline Equilibrate by extracting several times with 0 1*M* Tris-Cl, pH 8 0, and check that the pH is close to 8.0. Remove most of the overlying aqueous layer; add 240 mL of chloroform and 10 mL of isoamyl alcohol Mix and allow phases to separate Store refrigerated and protect from light
11 Chloroform
12 3*M* sodium acetate, pH 5 0 Dissolve sodium acetate in water and adjust pH to 5 0 with glacial acetic acid Adjust volume and treat with diethylpyrocarbonate (DEPC) to inactivate RNase To each liter of solution add 1 mL of DEPC; mix and incubate overnight at room temperature in a loosely capped bottle Autoclave to destroy residual DEPC
13 Absolute ethanol
14. Distilled water treated with DEPC, as described above

## 3. Method

### 3.1. Virus Purification

1 Collect 20 g of systemically infected leaf tissue displaying infection symptoms (*see* **Notes 3** and **4**). Using a pestle and mortar, with a little acid-washed sand to aid homogenization, grind the leaf tissue in 60 mL of virion extraction buffer (*see* **Note 5**). Start grinding with a small amount of buffer and progressively add more. Continue grinding until the tissue is well-macerated
2 Filter the homogenate through two layers of Miracloth into polypropylene centrifuge tubes. Squeeze as much liquid as possible out of material retained by the Miracloth without contaminating the filtrate with particulates.
3 Add butan-1-ol (0 8 mL/10 mL of filtrate) dropwise to the filtrate, while swirling the tube contents Cap the tubes and incubate at room temperature for 15 min

Every few minutes mix the tube contents by inversion Chlorophyll and coagulated material should collect in the upper organic phase

4 Centrifuge the tube contents at 10,000*g* for 30 min at 12°C Recover the lightly pigmented aqueous phase Avoid taking any of the pelleted material or the upper, organic layer If any of this undesired material is carried over, the aqueous phase should be centrifuged again in fresh tubes for 15 min Filter the clarified extract through two layers of Miracloth into fresh centrifuge tubes

5 Add 20% PEG solution to give a final concentration of 4% Mix the tube contents by inversion and incubate on ice for 15 min Periodically mix the tube contents The solution should turn cloudy as the virus precipitates.

6. Pellet the virus by centrifugation at 10,000*g* for 15 min at 4°C. This should yield a whitish pellet that may be contaminated with some traces of pigmented material Decant the supernatant and centrifuge briefly to collect residual liquid at the bottom of the tube Pipet off the residual liquid.

7. Dissolve the pellet in 8 mL of 10 m*M* phosphate buffer (*see* **Note 6**) Centrifuge at 10,000*g* for 15 min at 4°C Transfer the supernatant to a fresh tube

8 Add to the supernatant 1 7 mL of 5*M* NaCl and 2 42 mL of 20% PEG Mix tube contents and incubate on ice for fifteen min. Pellet the virus by centrifugation at 10,000*g* for 15 min at 4°C. This should yield a white viral pellet. Decant the supernatant, centrifuge briefly and pipet off the residual liquid

9. Dissolve the pellet in 2 mL of 10 m*M* phosphate buffer Divide the solution between two 1 5-mL microcentrifuge tubes Centrifuge the tubes in a microcentrifuge at 13,000*g* for 30 s and pipet the supernatants to fresh microcentrifuge tubes

10 This procedure should yield at least 20 mg of virus (*see* **Notes 7** and **8**) To determine the yields, prepare dilutions of small aliquots of the preparation and measure the absorbance at 260 and 280 nm Estimate the yield assuming an extinction coefficient ($E^{0.1\%}_{1\,cm}$) of three An $A_{260}/A_{280}$ ratio of about 1·19 is expected for TMV, however, this ratio varies between different tobamoviruses

## 3.2. RNA Extraction

1 Dilute an aliquot of the virion preparation to 10 mg/mL with 10 m*M* phosphate buffer Pipet 0 8 mL of this dilution to a 2-mL microcentrifuge tube and add 0 2 mL of 5X RNA extraction buffer Add 1 mL of phenol.chloroform, vortex briefly until an emulsion is formed, then centrifuge in a microcentrifuge tube at 13,000*g* for 5 min at room temperature.

2 Collect the upper aqueous phase without taking any of the denatured protein from the interface and transfer it to a fresh microcentrifuge tube Repeat the extraction with phenol.chloroform twice.

3 Collect the aqueous phase from the third phenol·chloroform extraction Add an equal volume of chloroform, and vortex briefly to form an emulsion Separate the phases by microcentrifugation.

4. Collect the upper aqueous phase, which should contain about 0 7 mL, and divide it equally between two 2-mL microcentrifuge tubes Add to each tube 0 1 vol of

3*M* sodium acetate and 2 5 vol of ethanol Mix and incubate the samples at –20°C for 15 min to precipitate the RNA

5 Pellet the RNA by microcentrifugation at 13,000*g* for 15 min at 4°C This should yield a visible white pellet. Decant the supernatant, centrifuge briefly to collect residual liquid at the bottom of the tubes and pipet this off without disturbing the pellet Dry the pellet under vacuum for a few minutes Add 0 2 mL of DEPC-treated water and dissolve the pellet by gently vortexing

6 This procedure should yield about 200 μg of RNA (*see* **Note 9**) Prepare dilutions of the dissolved RNA and measure the absorbance at 260 and 280 nm The preparation should have an $A_{260/280}$ ratio of 2 0 The RNA yield can be estimated by assuming that a 40 μg/mL solution of RNA has an absorbance of 1

## 4. Notes

1 Anyone using this protocol should familiarize themselves with the health and safety hazards related to 2-mercaptoethanol, butan-1-ol, diethylpyrocarbonate, phenol, 8-hydroxyquinoline, isoamyl alcohol, and chloroform All manipulations involving these chemicals should be carried out in a fume hood

2 If Miracloth is unavailable, muslin can be substituted However, muslin is less efficient at retaining particulates and its use is likely to lead to poorer purification

3 Before commencing purification, it is advisable to prepare a reasonably homogeneous viral population that has the desired phenotype This can be achieved by passaging the virus several times through a local lesion host at high viral dilutions, preparing inocula for each subsequent passage from individual lesions The final inoculum should be tested on a range of diagnostic host species before inoculation onto the host species from which the virus is to be purified

4 Choice of a propagation host is an important consideration in virus purification, and it is worthwhile testing a range of host plants in advance The choice of host plant is determined not only by the level of virus accumulation, but also by aspects of the host plant's biology Host plants that are not heavily lignified should be chosen to facilitate homogenization of host tissue, and hosts containing high levels of secondary products, which might interfere with the purification procedure, should be avoided The levels of virus that accumulate in the host plant are dependent on the growth conditions used; extremes of temperature and light should be avoided Hosts that the author has found useful for purification of the definitive tobamoviruses (tobacco mosaic virus, tomato mosaic virus, tobacco mild green mosaic virus, odontoglossum ringspot virus, ribgrass mosaic virus, and turnip vein clearing virus) include *Nicotiana benthamiana*, *N clevelandii*, and *N tabacum* cultivars

5. The initial steps of this purification process should be carried out at room temperature, because use of lower temperatures may result in precipitation of salts

6 Though the tobamoviruses are stable in a wide range of chemical environments and are thermotolerant, the particles are susceptible to fragmentation Therefore, dissolution of the viral pellet should be performed by gentle stirring with a teflon-coated rod, and the use of vortexers or tissue homogenizers, which produce high

shear forces, should be avoided. Dissolution of the viral pellet may prove difficult if large quantities of virus are present. If difficulties are encountered, the volume of buffer used for solvation should be increased

7 Ideally, the integrity of the purified virions should be confirmed by electron microscopic analysis. Samples of the virus can be stained with 2% sodium phosphotungstate, pH 7 0 The majority of particles should be 300 nm rods Depending on the tobamovirus, rods of one or two other discrete size classes may be visible These short rods result from the presence of encapsidated subgenomic RNAs

8 Difficulty in extracting TMV from some hosts has been reported and extraction has been facilitated by the use of the detergent Triton X-100. Another problem encountered during the purification process is adsorption of host components to the virus Methods involving adsorbents, such as charcoal, bentonite, and Celite, have been developed to eliminate this problem An alternative method for the purification of TMV, which makes use of Triton X-100 and Celite, has been described by Asselin and Zaitlin ***(16)***.

9 The integrity of the purified RNA should be checked by gel analysis A predominant band of 6.4 kb should be visible on denaturing gels. Minor bands may result from extracted subgenomic RNAs If high levels of RNA degradation are visible, this suggests that either the particles are fragmented or that there is RNase contamination The degree of particle fragmentation can be assessed as described above If RNase contamination is suspected more stringent procedures should be employed to avoid this Precautions should be taken to ensure that none of the plasticware used in the RNA extraction process is touched with bare hands and, when possible, solutions should be treated with DEPC Solutions containing Tris cannot be treated with DEPC, because it reacts with primary amines Therefore, it may be necessary to purchase molecular biology grade Tris, certified free of RNase, which can then be dissolved in DEPC-treated water

## References

1 *AAB descriptions of Plant Viruses*, Numbers 151 to 156, 184, 196, 330 and 351 Association of Applied Biologists, Warwick, UK.

2 Zimmern, D (1975) The 5' end group of tobacco mosaic virus RNA is $m^7G^{5'}ppp^{5'}Gp$ *Nucleic Acids Res* **2,** 1189–1201

3 Gallie, D R , Sleat, D E , Watts, J W., Turner, P. C , and Wilson, T M. A. (1987) The 5'-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo* *Nucleic Acids Res* **15,** 3257–3273

4 Goelet, P., Lomonossoff, G P , Butler, P J G , Akam, M E , Gait, M J , and Karn, J (1982) Nucleotide sequence of tobacco mosaic virus RNA *Proc Natl Acad Sci USA* **79,** 5818–5822

5 Young, N., Forney, J , and Zaitlin, M (1987) Tobacco mosaic virus replicase and replicative structures *J Cell Sci* **7 Suppl.,** 277–285

6 Pelham, H R B (1978) Leaky UAG termination codon in tobacco mosaic virus RNA. *Nature (London)* **272,** 469–471

7. Meshi, T., Watanabe, Y, Saito, T, Sugimoto, A M., Maeda, T., and Okada, Y (1987) Function of the 30 kDa protein of tobacco mosaic virus involvement in cell-to-cell movement and dispensability for replication *EMBO J* **6,** 2557–2563
8 Wolf, S , Deom, C M , Beachy, R N , and Lucas, W J (1989) Movement protein of tobacco mosaic virus modifies plasmodesmata size exclusion limit *Science* **246,** 377–379
9. Citovsky, V , Knorr, D , Schuster, G , and Zambryski, P (1990) The P30 movement protein of tobacco mosaic virus is a single strand nucleic acid binding protein *Cell* **60,** 637–647
10 Saito, T , Yamanaka, K , and Okada, Y (1990) Long-distance movement and viral assembly of tobacco mosaic virus mutants. *Virology* **176,** 329–336
11 Zimmern, D (1977) The nucleotide sequence at the origin for assembly on tobacco mosaic virus RNA *Cell* **11,** 463–482.
12 Leathers, V , Tanguay, R , Kobayashi, M , and Gallie, D R (1993) A phylogenetically conserved sequence within viral 3' untranslated RNA pseudoknots regulates translation. *Mol Cell Biol* **13,** 5331–5347
13. Stanley, W M (1935) Isolation of a crystalline protein possessing the properties of tobacco mosaic virus. *Science* **81,** 644,645
14. Gooding, G. V and Hebert, T T (1967) A simple technique for purification of tobacco mosaic virus in large quantities *Phytopathology* **57,** 1285
15 Leberman, R (1966) The isolation of plant viruses by means of "simple" coacervates *Virology* **30,** 341–347
16 Asselin, A and Zaitlin, M (1978) Characterization of a second protein asociated with virions of tobacco mosaic virus *Virology* **91,** 173–181

# 12

# Potexvirus Isolation and RNA Extraction

**Mounir G. AbouHaidar, Huimin Xu, and Kathleen L. Hefferon**

## 1. Introduction

The potexvirus family has at least 30 definitive and possible members, of which potato virus X (PVX) is the type member *(1–4)*. All potexviruses are morphologically similar, with flexuous, filamentous virions that range from 470 to 580 nm in length. Each virus particle contains a single-stranded, positive-sense RNA molecule, 5845–7015 nucleotides in length, which is encapsidated by 1000–1500 molecules of a single species of capsid protein (CP), with a mol wt ranging from 21 to 27 kDa *(5)* The particle has a helical structure, with 8.75 protein subunits per turn for papaya mosaic potexvirus (PMV) *(6)*. The genomic RNA (gRNA) contains a cap structure at the 5' terminus and is polyadenylated at the 3' terminus. The N- and C-terminal regions of the capsid proteins of potexviruses are quite variable *(7)*. The variability of amino acids at the N-terminus of potexvirus CP results in the low serological crossreactivity seen in potexviruses *(8)*. Individual potexviruses have mol wt in the order of $35 \times 10^6$ and sedimentation coefficients ranging from 100 to 130 S.

Five principal open reading frames (ORFs) have been identified in potexviruses (**Fig. 1**). ORF 1 encodes a protein that contains three amino acid sequence motifs characteristic of the conserved domains of methyltransferase, NTP-binding helicases, and RNA-dependent RNA polymerases *(5)*. ORFs 2–4 slightly overlap each other and are known as the triple gene block. The products of these three ORFs are all necessary for infectivity in the plant host, but may be dispensable for infection of protoplasts, and are believed to be involved in cell-to-cell movement of potexviruses *(9)*. ORF 5 is the 3'-terminal ORF and encodes the CP.

Genomic RNA of potexviruses is believed to be functionally monocistronic, and only ORF 1 protein can be translated directly from the gRNA *(10,11)*.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

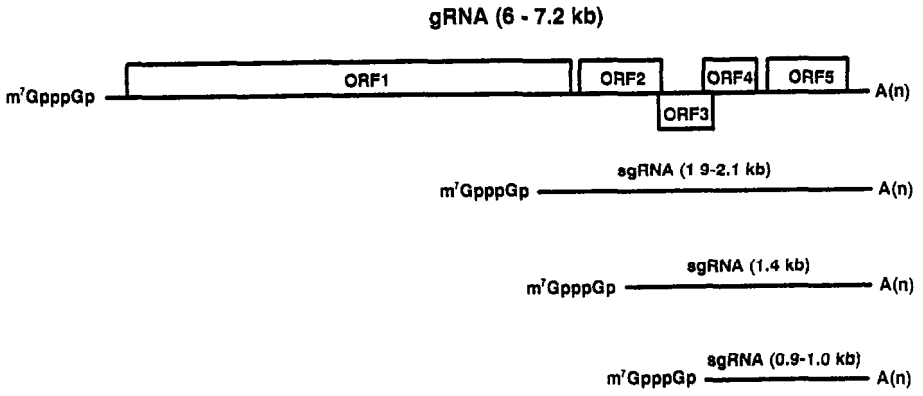



Fig 1 General organization of a potexvirus genome and its subgenomic RNAs Genomic organization of PVX, the type member of the potexviruses Three subgenomic RNAs (sgRNAs) are shown below genomic RNA (gRNA) A(n) indicates the poly(A) tails at the 3' end of gRNA and sgRNAs. $m^7GpppGp$ represents the cap structure at the 5' end of both genomic and subgenomic RNAs ORF1–ORF5 correspond to the five ORFs ORF5 encodes the coat protein Sizes of RNA in kb are indicated in parentheses

However, recent information indicates that the CP of PVX could be expressed in vivo from a dicistronic message *(51)*. In addition to the gRNA, several subgenomic RNAs (sgRNAs) of 0.9, 1.4, and 2.1 kb in length have been detected from plants infected with potexviruses (*12–15*, **Fig. 1**) These sgRNAs are capped and polyadenylated like the gRNA *(13,16,17)*. The 5' ends of potexviral sgRNAs correspond to internal genomic regions; the 3' ends are coterminal with the genomic RNAs *(13)*. The results of in vitro translation of sgRNAs suggest that the 25-kDa protein (ORF2) of PVX is expressed as a single translation product of the 2.1 kb sgRNA, both 12-kDa (ORF3) and 8-kDa (ORF4) proteins are expressed from the same 1 4-kb sgRNA, which appears to be functionally bicistronic *(18)*. The CP can be efficiently translated from the 0.9 kb sgRNA *(19)*.

## 2. Materials

### 2.1. Virus Purification

1. 0.1*M* Phosphate buffer, pH 7 2
2. 0.1*M* Tris-borate acid buffer, pH 7 5
3. *n*-Butanol
4. Polyethylene glycol (PEG), mol wt 8000
5. Sodium chloride
6. Sucrose cushion 30% Sucrose in 0 1*M* Tris-borate acid buffer, pH 7 5 (w/v).

7 Cesium chloride (CsCl) density gradient 10 g of CsCl is mixed with 20 mL of virus solution to reach a density of 1 3667
8 Sodium azide.

### 2.2. Viral gRNA Extraction

1 10X DNase I reaction buffer (1X buffer = 20 m*M* Tris-HCl, pH 8 3, 50 m*M* KCl, 2 5 m*M* $MgCl_2$, 100 µg/mL of acetylated bovine serum albumin [BSA])
2 Deoxyribonuclease I (DNase I)
3. Sodium dodecyl sulfate (SDS), 10%.
4 TE buffer 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7 5
5 Phenol· The phenol used is pre-equilibrated with 0 1*M* Tris-HCl, pH 8 0, containing 0 2% β-mercaptoethanol
6 Chloroform
7. 70 and 95% Ethanol
8. Diethyl pyrocarbonate-treated distilled water (DEPC-$dH_2O$) DEPC (0 5 mL) is dissolved in 2 mL 95% ethanol, and then mixed with 1 L $dH_2O$ The mixture is stored at 4°C overnight before being autoclaved
9 RNase-free glassware
10 Sodium acetate
11 Triton X-100.

### 2.3. Extraction of Viral or Polyribosomal RNAs from Infected Plant Tissue

1 Oligo(dT)-cellulose
2 Loading buffer A. 20 m*M* Tris-HCl, pH 7 5, 0.5*M* LiCl, 1 m*M* EDTA, 0 1% SDS
3 Loading buffer B 20 m*M* Tris-HCl, pH 7 5, 0 1*M* LiCl, 1 m*M* EDTA, 0 1% SDS
4 Solution A 0 1*N* NaOH, 5 m*M* EDTA
5 Solution B 10 m*M* Tris-HCl, pH 7 5, 1 m*M* EDTA, 0 05% SDS
6 Extraction buffer A 200 m*M* Tris-HCl, pH 9 0, 400 m*M* LiCl, 25 m*M* EDTA, 1% SDS, all in DEPC-$dH_2O$
7. Extraction buffer B 200 m*M* Tris-HCl, pH 8 5, 400 m*M* KCl, 200 m*M* sucrose, 35 m*M* $MgCl_2$, 25 m*M* EDTA, and 1% β-mercaptoethanol
8 Sucrose cushion: 60% sucrose in 40 m*M* Tris-HCl, pH 8 5, 200 m*M* KCl, 30 m*M* $MgCl_2$, and 5 m*M* EDTA
9 Buffer A: 100 m*M* Tris-HCl, pH 7 5, 1*M* KCl, 10 m*M* $MgCl_2$, and 2 5 m*M* puromycin.
10. Buffer B· 50 m*M* Tris-HCl, pH 7 5, 500 m*M* KCl, 5 m*M* $MgCl_2$
11 Liquid nitrogen

## 3. Methods

### 3.1. Purification of Virus

Many potexviruses occur in high concentration in their host plants and are relatively stable in extracted leaf sap. These properties make it possible to develop protocols for the purification of most potexviruses with high virus

**Table 1**
**Propagation Host Species of Some Potexviruses**

| Potexviruses | Propagation species | Refs for purification methods |
|---|---|---|
| Bamboo mosaic (BaMV) | *Hordeum vulgare* | *34* |
| Cactus X (CVX) | *Chenopodium quinoa* | *35* |
| Cassava common mosaic (CCMV) | *Euphorbia prunifolia* | *36* |
| Clover yellow mosaic (CYMV) | *Vicia faba* | *37* |
| Cymbidium mosaic (CybMV) | *Datura stramonium* | *38* |
| Dioscorea latent (DLV) | *Nicotiana megalosiphon* | *39* |
| Foxtail mosaic (FMV) | *H vulgare* | *37* |
| Hippeastrum latent (HsLV) | *Hippeastrum hybridum* | *40* |
| Hydrangea ringspot (HRSV) | *Hydrangea macrophylla* | *41* |
| Lily virus X (LVX) | *Lilium hybrid* | *42* |
| Nandina mosaic (NdMV) | *N benthamiana* | *43* |
| Narcissus mosaic (NMV) | *Narcissus tazetta* | *44* |
| Nerine virus X (NVX) | *Nerine sarniensis* | *45* |
| Papaya mosaic (PMV) | *Carica papaya* | *22* |
| Pepino mosaic (PpMV) | *N glutinosa* | *46* |
| Plantago asiatica mosaic (PlAMV) | *Plantago asiatica* | *47* |
| Potato aucuba mosaic (PAMV) | *N tabacum* cv Xanthi nc | *32* |
| Potato X (PVX) | *N tabacum* | *22,23* |
| Strawberry mild yellow edge-associated (SMYEAV) | *Rubus rosifolius* | *31* |
| Viola mottle (VMV) | *C quinoa* | *21* |
| White clover mosaic (WClMV) | *Phaseolus vulgaris* | *48* |
| Wineberry latent (WbLV) | *C quinoa* | *49* |
| Zygocactus X (ZVX) | *C quinoa* | *50* |

yields. Approximately 0.25–3 g/kg tissue of purified virus preparations can be obtained for most potexviruses (*20,21*, *see* **Note 1**). To purify different potexviruses, a variety of methods have been developed A potexvirus is inoculated onto an appropriate propagation host (**Table 1**). Approximately 10–14 d postinoculation, the infected leaf tissues can be collected and homogenized in a suitable buffer. Clarification of the sap is achieved by passing through cheesecloth, by filtration/absorption, by treatment with organic solvents, or by low-speed centrifugation. The virus can be concentrated either by differential centrifugation, or by precipitation with PEG. Further purification of the virus can be achieved by density gradient centrifugation in sucrose or in CsCl.

The purification method described here is based on PVX, according to Erickson and Bancroft *(22)* and Huisman et al. *(23)*.

1. *Nicotiana tabacum* cv Samsun is a good propagation host for PVX. The plants (four true-leaf-age) are lightly dusted with Carborundum (400-mesh), and inoculated with either purified PVX (1 μg/mL or higher concentration) or infected leaf extracts (*see* **Note 2**) The inoculated plants are kept under greenhouse condition (25°C, 16-h photoperiod) Two weeks postinoculation, the leaves of inoculated plants can be collected for virus purification (*see* **Note 2**)
2. Homogenize infected leaves in Tris-borate buffer containing 0 2% β-mercaptoethanol (1–2 mL/g of leaves). The subsequent steps were performed at 4°C (*see* **Note 3**)
3. Squeeze the homogenized tissue through four layers of cheesecloth Add *n*-butanol to the sap to a final concentration of 6%
4. Keep the mixture on ice for 45 min with constant stirring Centrifuge for 10 min at 15,000*g* and save the supernatant
5. Precipitate the virus from the supernatant by the addition of PEG 8000, to a final concentration of 8% in the presence of 2% NaCl, and keep the mixture at 4°C for 30–60 min
6. Centrifuge at 15,000*g* for 10 min, resuspend the pellets in Tris-borate buffer, pH 7 5, overnight at 4°C without vortexing. The virus solution is then centrifuged three times at 7500*g* for 5 min each. Keep the supernatant
7. Overlay the virus solution onto 4 mL of sucrose cushion in Ti 60 ultracentrifuge tubes (Beckman) (*see* **Note 4**) Pellet the virus at 86,500*g* for 3 h at 4°C Pellets are redissolved, in the same buffer as above, overnight at 4°C, and centrifuged three times at 7500*g* for 10 min each (*see* **Note 5**)
8. The virus in the supernatant is then centrifuged in a CsCl density gradient for 17 h at 86,500*g* (15°C) The virus bands (white opalescent seen with light from beneath against a black object or in a dark room) are collected and diluted four times with Tris-borate buffer A CsCl purification is only required for ultrapure preparations of the virus, otherwise, go to **step 10**.
9. The virus is then collected by centrifugation at 100,000*g* for 2 h. Pellets are redissolved in the same buffer overnight.
10. Optical density (OD) readings are taken at 260 and 280 nm to determine the virus purity ($A_{260}/A_{280}$ ratio of 1 2 for PVX) and concentration using extinction coefficient 260 nm ($E^{260\ nm}_{0\ 1\%,\ 1\ cm}$ = 3 0 for PVX) ***(1)***. The yield is approx 0 5 to 1 0 g/kg leaf tissues The virus particles can be negatively stained with 1% uranyl acetate and examined by transmission electron microscopy
11. Keep the purified virus preparations at 4°C in the presence of 0 1% sodium azide. Under these conditions, the virus remains infectious for at least 3 yr

## 3.2. Extraction of Viral gRNA and sgRNA

### 3.2.1. Extraction of Viral RNA from Purified Virions

In general, to extract viral gRNA from the purified virions, the virus is treated with SDS to strip off the capsid protein. The capsid protein is then removed by extraction with phenol. Chloroform is used to remove the phenol,

and the RNA can be precipitated from the aqueous layer with ethanol in the presence of sodium acetate Host DNA left in purified virus preparations will also be carried over to RNA extraction. This usually causes some problems for further cDNA synthesis and cloning experiments. Therefore, the host DNA must be removed using deoxyribonucleases (DNase) before RNA extraction. Another problem during the isolation of viral gRNA is the degradation of RNA by nucleases, mainly RNase A. To minimize RNA degradation, all tubes, tips, containers, and glasswares should be washed in DEPC-$dH_2O$, and the glasswares are further baked for more than 6 h at 160°C (*see* **Note 6**) To obtain full-length gRNA, which is of great importance in cloning full-length cDNAs, the viral RNA should be centrifuged in a sucrose gradient

Host DNA contaminating the virus preparations may be removed before the isolation of RNA from the purified virions as follows

1 To 1 mL of virus solution, add 100 µL of DNase I reaction buffer (10X) and 5 U of DNase I Keep the mixture at 37°C for 30 min and terminate the reaction with addition of EDTA to 5 m*M*
2 Centrifuge the DNase treated virus at 86,500*g* for 2 h and dissolve the virus pellets in DEPC-$dH_2O$

Viral gRNA can be extracted according to the methods of AbouHaidar and Bancroft ***(24)***, and Maniatis et al. ***(25)***. Bamboo mosaic virus has been found to have a satellite RNA (sRNA), which is also encapsidated by CP, and the sRNA can also be extracted from purified virions (**ref. *20***, *see* **Note 7**).

1 Add SDS to the DNase I-treated purified virus solution, to a final concentration of 0 1% (w/v).
2 Add 2 vol of phenol·chloroform (1 1 v/v), keep the mixture at 40°C for 5–10 min, with occasional vortexing
3 Centrifuge at 12,500*g* for 5 min at room temperature and transfer the aqueous phase to a new tube
4. Add DEPC-$dH_2O$ (0.1 vol) to the phenol·chloroform phase, vortex, and centrifuge as above Collect the aqueous phase
5 Combine the two aqueous phases. Extract the aqueous phase with an equal volume of phenol chloroform and centrifuge as above.
6 Transfer the aqueous layer to a new tube and subsequently re-extract twice with 2 vol of chloroform/isoamyl alcohol (24 1, v/v)
7 Precipitate the RNA by the addition of sodium acetate to a final concentration of 0 25*M* and 2 5 vol of ice-cold 95% ethanol.
8 Keep the mixture at –70°C for 20 min (or –20°C overnight) The viral RNA is then collected by centrifugation at 12,500*g* for 10 min
9. Rinse the RNA pellets with 70% ethanol, vacuum-dry the RNA pellet for 5–8 min, and dissolve in a desired volume of DEPC-$dH_2O$ (or TE buffer) (*see* **Note 8**)

10. The purity and concentration of the RNA can be measured according to the OD readings at 260 and 280 nm The RNA preparation can also be analyzed by electrophoresis on agarose gel A ratio of $OD_{260}/OD_{280} \geq 1\ 9$ indicates that the RNA is essentially free from protein

An extinction coefficient $E^{260\ nm}_{0\ 1\%,\ 1\ cm} = 25$ is used to quantify the amount of RNA. To obtain high-quality, full-length, infectious gRNA, the RNA extracts should be further centrifuged in 5–20% (w/v) RNase-free sucrose gradient in 10 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, as follows·

1 Load the RNA solution onto a continuous 5–20% sucrose gradient and centrifuge at 87,800*g* for 13 h in a swing-bucket rotor at 4°C.
2 Pass the gradient through a UV scanner-fractionator
3 Collect fractions corresponding to the full-length gRNA, precipitate RNA with 2 vol of 95% ethanol at –20°C overnight and recover RNA by centrifugation for 10 min at 12,500*g* Wash RNA pellets with a large volume of 70% ethanol

### *3 2.2. Extraction of Viral RNA from Infected Plant Tissue*

This technique can be used for extracting viral genomic and subgenomic RNAs from infected tissues or from transgenic plants expressing viral sequences. Total plant RNA is isolated by grinding frozen plant tissue in extraction buffer, extracting with phenol, and precipitating with ethanol. RNA quantity can be determined by UV absorption or by electrophoresis on agarose gels. Viral RNA can be identified from plant RNA by Northern blot analysis (*see* **Note 9**).

A variety of procedures can be used to isolate viral RNA from infected plant tissues. These include extraction in a phenol–cresol solution ***(26)*** and extraction with hot phenol–SDS ***(27)*** The highest yield of PVX RNA was achieved in the following manner:

1 Place leaves (0.4 g) in a prechilled mortar and pestle and grind to a powder in liquid nitrogen. Add 6 mL of extraction buffer A and allow to freeze.
2 Add 6 mL Tris-saturated phenol, pH 8 0, and DEPC (to a final concentration of 1%), and thaw the mixture at room temperature for 10 min Pour the slurry into a tube and maintain on ice (*see* **Note 10**)
3 Remove large debris by centrifugation at 12,000*g* for 10 min at 4°C Extract supernatant with phenol, then twice with 1 vol phenol.chloroform (1 1), and twice with chloroform. Transfer the aqueous phase to a new tube and precipitate RNA by adding 2 vol 95% ethanol and storing at –20°C for 1 h. Pellet RNA by centrifugation at 5800*g* for 20 min Wash RNA in 70% ethanol, vacuum-dry for 15 min, and resuspend in 1 mL TE buffer
4. Viral RNA can be identified from cellular RNAs by electrophoresis on a 1 5% agarose gel containing 10% formaldehyde, and by Northern blot analysis.

### 3.2.3 Selection of Poly(A)$^+$ mRNA

If the proportion of viral RNA is very low compared to plant total RNA, it may be helpful to select for viral and plant mRNAs using an oligo(dT)-cellulose column. This is a modification of the method of Guilford and Forster ***(14)***, and is performed as follows:

1. Add oligo(dT)-cellulose to loading buffer A to make a final concentration of 0.5 mg/mL. Pour 1 mL of this solution into a sterile siliconized Pasteur pipet plugged with siliconized glass wool (*see* **Note 11**).
2. Wash the column in 3 vol each of DEPC-$dH_2O$ and solution A. Test the effluent to ensure that the pH of the column is maintained between 6.0 and 8.0. Wash column with 5 vol of loading buffer A.
3. Heat RNA sample at 65°C for 5 min and add an equal volume of loading buffer A. Cool the mixture to room temperature and apply to the column.
4. Wash the column in 4–6 vol of loading buffer A, then in another 4–6 vol of loading buffer B.
5. Elute poly(A)$^+$ RNA with 3 vol of solution B. Add 3*M* sodium acetate, pH 5.2 (final concentration of 0.3*M*), to elute RNA. Add 2.5 vol 95% ethanol to precipitate RNA, and store at –20°C.
6. Pellet the RNA by centrifugation at 5800*g* at 4°C and resuspend in TE buffer. Determine RNA concentration by UV absorption.

### 3.2.4. Isolation of Polyribosomal RNAs

Translationally active RNAs can be isolated by extracting polysomes and purifying them on a sucrose cushion. The ribosomes remain tightly bound to the mRNA by incubation with puromycin, and polysomes can be isolated by centrifugation on a sucrose gradient. Polysomal RNAs were isolated by the method of Palukaitis ***(28)***.

1. Freeze 2 g of leaf tissue in liquid nitrogen and grind to a powder in a prechilled mortar. Resuspend powder in 3 mL extraction buffer B (*see* **Note 10**).
2. Remove large debris by centrifugation at 12,000*g* for 24 min at 4°C and add Triton X-100 to the supernatant to a final concentration of 1%. Pellet polyribosomes on a 4-mL sucrose cushion by centrifugation at 100,000*g* for 90 min at 4°C.
3. Remove sucrose solution with an aspirator, wash the pellet with 1 mL of water, and store at –20°C until use.
4. Resuspend pellets in 0.2 mL of $dH_2O$ and 0.25 mL of buffer A. Incubate mixture on ice for 15 min, then at 37°C for 10 min.
5. Layer mixture onto a 5–20% (w/v) sucrose gradient in buffer B and centrifuge at 4°C for 4 h at 100,000*g* (*see* **Note 12**).
6. Collect fractions (0.5 mL) on a density gradient fractionator and determine the RNA concentration of each sample by UV absorption. The first peak (top of the gradient) identified contains mostly tRNA, the middle peak(s) contains mRNA

followed by rRNA, and the final peak corresponds to viral RNA. Phenol-extract and ethanol-precipitate each fraction. Pellet RNA and resuspend in TE buffer.

7. Load one-half of each fraction onto a 1.5% agarose gel containing 10% formaldehyde and identify viral RNA by Northern blot analysis

## 4. Notes

1. It is usually easy to purify those definitive members of potexviruses, most of the possible members, especially those potexviruses that only infect woody hosts, have not been successfully purified, and some purification attempts have failed *(29)* In those cases, virus replicative nucleic acids (dsRNAs) can be extracted from virus infected tissues and used as templates for gene cloning *(30,31)* and RNA analysis
2. If the infected tissues are used as inoculum, the tissues are ground in 0 1*M* phosphate buffer, pH 7.2 (2 mL buffer/1 g tissue), and centrifuged briefly (10,000*g* for 4 min) before the extracts are used for inoculation Virus-infected plants will be ready for virus purification 10–14 d postinoculation Infected leaves can also be stored at –20°C Both fresh and frozen leaf tissue can be used for virus purification
3. For most potexviruses, pretreatment of the infected tissues is usually not necessary In the case of potato aucuba mosaic virus (PAMV), however, infiltrating the tissues with extraction buffer under vacuum before homogenizing them is believed to be helpful *(32)*
4. It is recommended that centrifuge tubes be filled first with the virus solution and then that the sucrose cushion be deposited at the bottom of each tube with a Pasteur pipet
5. The virions of potexviruses have a tendency to aggregate side-to-side or end-to-end and to break This will result in a lower yield of purified virus and difficulties in the isolation of full-length gRNA To avoid or minimize these problems, it is recommended that during purification, after each centrifugation step, the pellets be covered with buffer at 4°C for a prolonged period (e g , overnight) before complete resuspension It is also recommended to dissolve the pellets by gentle repeated pipeting and not by vortexing Many potexviruses (e g., papaya mosaic virus) may have a high yield of virus It is recommended to dilute the virus preparations prior to ultracentrifugation. High concentrations of the virus result in increased viscosity and prolonged ultracentrifugation time and/or loss of yield Pellets of diluted virus preparation are generally cleaner.
6. It is essential in the steps involving the handling of RNA that all glasswares and solutions are RNase-free This can be accomplished by baking glasswares at 160°C for at least 6 h and making all solutions with DEPC-$dH_2O$
7. Bamboo mosaic virus is the only potexvirus to contain a packaged satellite RNA (sRNA) Both gRNA and sRNAs can be extracted from purified virions and separated by electrophoresis in nondenaturing 1% low-melting agarose gel After electrophoresis, both RNAs are then isolated from gel slices, followed by phenol extraction and ethanol precipitation *(33)*

8 RNA pellets can be dissolved in DEPC-$dH_2O$ (or RNase-free TE buffer) and the solution of isolated viral gRNA is kept at –20°C If the RNA is not used immediately after isolation, it is recommended to keep the RNA under ethanol at –20°C or in the form of dried RNA pellets at –20°C

9 To synthesize viral-specific cDNA probes, primers (either random primer or synthetic viral-specific primers) are annealed to the viral RNAs (gRNA, sgRNA, or RNA fragment) The cDNA strand is synthesized by reverse transcriptase using viral RNA as templates in the presence of dCTP, dGTP, dTTP, and $\alpha^{32}$P-dATP (or biotin-14-dATP) Unincorporated nucleotides can be removed by passing the reaction mixture through a Sephadex G50 column

10 When extracting RNA from leaf tissue, make sure that the tissue, extraction buffer, and phenol are frozen in liquid nitrogen Proceed with experiment immediately after thawing to avoid any loss of RNA through degradation Make sure plant extract and all solutions are kept on ice

11 Glass wool was soaked in dimethyldichlorosilane for 30 min at room temperature under a fume hood Afterward, glass wool was removed with a forceps, air-dried on Whatman 3MM paper, and was ready for use

12 It may be necessary to decrease the speed and duration of centrifugation, depending on the potexvirus used

## References

1 Bercks, R (1970) Potato virus X *CMI/AAB Descrip Plant Vir* **No. 4**

2 Francki, R I B , Milne, R G , and Hatta, T (1985) Potexvirus group in *Atlas of Plant Viruses*, vol II (Francki, R I B , Milne, R G , and Hatta, T , eds ), CRC, Boca Raton, FL, pp 159–172

3. Koenig, R and Lesemann, D -E (1978) Potexvirus group. *CMI/AAB Descrip Plant Vir* **No. 200**

4 Purcifull, D E. and Edwardson, J R (1981) Potexviruses, in *Handbook of Plant Virus Infections and Comparative Diagnosis* (Kurstak, E , ed ), Elsevier/North-Holland, Amsterdam, pp 627–693

5 Xu, H , Leclerc, D , Leung, B , and AbouHaidar, M G (1994) The entire nucleotide sequence and genomic organization of potato aucuba mosaic virus *Arch Virol* **135,** 461–469

6 Tollin, P , Bancroft, J B , Richardson, J F , Payne, N C , and Beveridge, T J (1979) Diffraction studies of papaya mosaic virus. *Virology* **5,** 108–115

7 AbouHaidar, M G and Lai, R (1989) Nucleotide sequence of the 3'-terminal region of clover yellow mosaic virus RNA *J Gen Virol* **70,** 1871–1875

8 Koenig, R and Torrance, L (1986) Antigenic analysis of potato virus X by means of monoclonal antibodies *J Gen Virol* **67,** 2145–2151

9 Beck, D L , Guilford, P J., Voot, D M., Andersen, M T , and Forster, R. L S (1991) Triple gene block proteins of white clover mosaic potexvirus are required for transport *Virology* **183,** 695–702

10 Miroshnichenko, N A , Karpova, O V , Morozov, S Yu , Rodionova, N P , and Atabekov, J G (1988) Translation arrest of potato virus X RNA in Krebs-2-cell-

free system. RNase H cleavage promoted by complementary oligodeoxynucleotides *FEBS Lett* **234,** 65–68

11 Skryabin, K G , Morozov, S. Yu., Kraev, A. S., Rozanov, M N , Chernov, B. K , Lukasheva, L I , and Atabekov, J G (1988) Conserved and variable elements in RNA genome of potexviruses *FEBS Lett* **24,** 33–40

12 Bendena, W G , Bancroft, J B , and Mackie, G A. (1987) Molecular cloning of clover yellow mosaic virus RNA identification of coat proteins coding sequences in vivo and in vitro *Virology* **157,** 276–284

13 Dolja, V V., Grama, D P , Morozov, S. Yu , and Atabekov, J G (1987) Potato virus X-related single- and double-stranded RNAs· characterization and identification of terminal structures *FEBS Lett* **214,** 308–312

14 Guilford, P J and Forster, R L S (1986) Detection of polyadenylated subgenomic RNAs in leaves infected with the potexvirus daphne virus X *J Gen Virol* **67,** 83–90.

15 Mackie, G A , Johnston, R , and Bancroft, J. B. (1988) Single- and double-stranded viral RNAs in plants infected with potexviruses papaya mosaic virus and foxtail mosaic virus. *Intervirology* **29,** 170–177.

16 Bancroft, J B , Rouleau, M , Johnston, R , Prins, L , and Mackie, G A (1991) The entire nucleotide sequence of foxtail mosaic virus RNA. *J Gen Virol* **72,** 2173–2181

17 White, K A and Mackie, G A (1990) Control and expression of 3' open reading frames in clover yellow mosaic virus *Virology* **179,** 576–584

18 Morozov, S Yu , Miroshnichenko, N A , Solovyev, A G , Fedorkin, O N , Zelenina, D A , Lukasheva, L. I., Karasev, A. V., Dolja, V. V., and Atabekov, J. G. (1991) Expression strategy of the potato virus X triple gene block *J Gen Virol* **72,** 2039–2042

19 Karasev, A V, Alexandrova, N M , Miroshnichenko, N A , Nesterova, I V , and Dorokhov, Yu. L (1987) Translation activity of messenger RNAs from potato virus X-infected plants. *Mol Biol* **21,** 1367–1377

20 Lin, M T , Kitajima, E W , Cupertino, F P , and Costa, C L (1977) Partial purification and some properties of bamboo mosaic virus *Phytopathology* **67,** 1439–1443

21 Lisa, V and Dellavalle, G (1977) Viola mottle virus, a new member of the potexvirus group *Phytopathol Zeits* **89,** 82–89

22 Erickson, J W and Bancroft, J B (1978) The self-assembly of papaya mosaic virus *Virology* **90,** 36–46

23 Huisman, M J., Linthorst, H. M , Bol, J., and Cornelissen, B J. C (1988) The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *J Gen Virol* **69,** 1789–1798

24 AbouHaidar, M. G. and Bancroft, J. B. (1978) The initiation of papaya mosaic virus assembly. *Virology* **90,** 54–59

25 Maniatis, T., Fritsch, E F , and Sambrook, J (1982) *Molecular Cloning A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

26 Siegel, A , Zaitlin, M , and Duda, C T (1973) Replication of tobacco mosaic virus IV Further characterization of viral-related RNAs *Virology* **53,** 75–83

27. Hunter, T R , Hunt, T , Knowland, J , and Zimmern, D (1976) Messenger RNA for the coat protein of tobacco mosaic virus *Nature* **260,** 759–764
28 Palukaitis, P. (1984) Detection and characterization of subgenomic RNA in plant viruses *Methods Virol* **7,** 259–317
29 Stace-Smith, R and Martin, R R (1993) Virus purification in relation to diagnosis, in *Diagnosis of Plant Virus Diseases* (Matthews, R E F , ed.), CRC, London, pp 129–158
30. Jelkmann, W , Martin, R R , and Maiss, E (1989) Cloning of four plant viruses from small quantities of double-stranded RNA *Phytopathology* **79,** 1250–1253
31 Martin, R R , Jelkmann, W , Spiegel, S , and Converse, R H (1989) Molecular cloning of the dsRNA associated with strawberry mild yellow-edge virus *Acta Hort.* **236,** 111–116.
32 Kassanis, B and Govier, D A (1972) Potato aucuba mosaic virus *CMI/AAB Descrip Plant Vir* **No. 98**
33 Lin, N S and Hsu, Y H (1994) A satellite RNA associated with bamboo mosaic potexvirus *Virology* **202,** 707–714.
34 Lin, N S and Chen, C C (1991) Association of bamboo mosaic virus (BaMV) and BaMV-specific electro-dense crystalline bodies with chloroplasts *Phytopathology* **81,** 1551–1555
35. Bercks, R (1971) Cactus virus X *CMI/AAB Descrip Plant Vir* **No. 58**
36. Costa, A. S and Kitajima, E. W (1972) Cassava common mosaic virus *CMI/AAB Descrip Plant Vir* **No. 90**
37 Bancroft, J B , AbouHaidar, M G., and Erickson, J W (1979) The assembly of clover yellow mosaic virus and its protein *Virology* **98,** 121–130.
38 Frowd, J A and Tremaine, J H (1977) Physical, chemical and serological properties of cymbidium mosaic virus *Phytopathology* **67,** 43–49
39 Phillips, S , Piggott, J D'A , and Brunt, A A (1986) Further evidence that dioscorea latent is a potexvirus *Ann Appl Biol* **109,** 137–145
40 Brolman-Hupkes, J. E (1975) Tentative description of hippeastrum latent virus in Hippeastrum hybridum *Neth J Plant Pathol* **81,** 226–236.
41 Koenig, R (1973) Hydrangea ringspot virus *CMI/AAB Descrip Plant Vir* **No. 114.**
42 Dercks, A F L M and Vink-van den Abeele, J L (1980) Purification of lily symptomless virus Use and value of antisera against intact and pyrrolidine-degraded virus for testing lily and tulips *Neth J Plant Pathol* **86,** 239–250
43 Zettler, F. W., Hiebert, E , Maciel-Zambolim, E , Christie, R G , and Abo El-Nil, M M (1980) A potexvirus infecting nandina domestica "Harbor Dwarf" *Acta Hort* **110,** 71–80
44 Zuidema, D , Linthorst, H J M , Huisman, M J , Asjes, C J , and Bol, J F (1989) Nucleotide sequence of narcissus mosaic virus RNA *J Gen Virol* **70,** 267–276.
45 Maat, D Z (1976) Two potexviruses in Nerine *Neth J Plant Pathol* **82,** 95–102
46 Jones, R A. C., Koenig, R., and Lesemann, D -E. (1980) Pepino mosaic virus, a new potexvirus from pepino (*Solanum muricatum*) *Ann App Biol* **94,** 61–68

47 Solovyev, A G , Novikov, V. K , Merits, A , Savenkov, E I , Zelenina, D A , Tyulkina, L. G , and Morozov, S Yu (1994) Genome characterization and taxonomy of *Plantago asiatica* mosaic potexvirus *J Gen Virol* **75,** 259–267
48 Bercks, R (1971) White clover mosaic virus *CMI/AAB Descrip Plant Vir* **No. 41.**
49 Jones, A T (1977) Partial purification and some properties of wineberry latent, a virus obtained from *Rubus phoenicolasius Ann App Biol* **86,** 199–208
50. Giri, L and Chessin, M (1975) Zygocactus virus X. *Phytopathol Zeits* **83,** 40–48.
51. Hefferon, K L , Khalilian H , Xu, H., and AbouHaidar, M. G (1997) Expression of the coat protein of potato virus X from a dicistronic mRNA in transgenic potato plants *J Gen Virol* **78,** in press

# 13

# Carlavirus Isolation and RNA Extraction

**Gary D. Foster**

## 1. Introduction

All members of the genus are known to be transmitted mechanically, with the majority also being transmitted in a nonpersistent manner by aphids *(1)*, though one confirmed carlavirus is known to be transmitted by whiteflies *(2)*. Carlaviruses are noted for their narrow host range and tendency to induce little or no symptoms. This has led to many of the common names of carlaviruses, including carnation latent (CLV), American hop latent (AHLV), and lily symptomless virus (LSV). Although most carlaviruses do cause mild symptoms, there are a number of viruses that cause serious diseases on their own, for example, potato virus S (PVS) and M (PVM), blueberry scorch virus (BBScV), poplar mosaic virus (PMV), and a number of others that cause serious disease in mixed viral infections (with other viruses).

The virus particles of carlaviruses are slightly flexuous, with a typical length of 660 nm and diameter of 12 nm *(1)*. These particles, which consist of a single species of protein of ca. 34 kDa organized with helical symmetry, have sedimentation coefficients of approx 157 *S*, a buoyant density in CsCl of 1.322 g/cm$^3$, an extinction coefficient at 260 nm of 2.88 cm$^2$/mg$^1$, and a nucleic acid content of ca. 6% *(1,3)*.

The genomic RNA of a range of carlaviruses has been estimated by agarose gel analysis to be in the size range of 7.3–7.7 kb. However, the recent reports of two full-length genomic RNA sequences have indicated a genome size of 8534 nucleotides for PV*M (4)*, and 8512 for BBScV *(5)*. In addition, a wide range of carlaviruses have been sequenced in their 3' terminal region, including PVS, HeLVS, CLV, LSV, chrysanthemum virus B, and cowpea mild mottle virus *(2)*. All show a similar genome organization, with similar sized open reading

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

frames, and a high level of homology in the amino acid sequences between corresponding proteins

Only the replicase ORF (approx 223 kDa) is translated from the full-length genomic RNA In vitro translation data from a range of carlaviruses suggested that this large ORF was proteolytically processed, with approx 30–40 kDa being removed Recent elegant experiments by Lawrence et al. ***(8)***, using in vitro transcribed and translated replicase ORF and full-length infectious clones of BBScV, have clearly demonstrated that this is indeed the case

The 3' terminal ORFs appear to be translated from two subgenomic mRNAs, which can be found in infected tissue, and, for some viruses, can be detected within purified virus preparations ***(9,10)***. The 3' ORFs encode proteins of 25, 12, and 7 kDa, which have been designated the triple gene block, the 34 kDa coat protein, and an ORF of approx 11 kDa, present at the 3' terminus, which is unique to carlaviruses ***(1)*** The sizes of reported RNAs and typical ORFs for carlaviruses are summarized in **Fig. 1**, with the position of the 34-kDa coat protein (CP) indicated.

## 2. Materials

### 2.1. Virus Isolation

1 0 5*M* borate buffer, pH 7 8, with 5 m*M* EDTA
2 Diethyl ether
3 Polyethylene glycol (PEG), average mol wt 8000
4. Thioglycolic acid
5. Triton X-100
6. Cesium chloride
7 Sterile $dH_2O$
8 High-capacity (500 mL) centrifuge, capable of 10,000*g*
9 Ultracentrifuge capable of 110,000*g*
10 Blender
11 Stir plate and magnetic stirrers

### 2.2. RNA Extraction

RNA dissociation buffer, TE buffer, and 3*M* sodium acetate should be autoclaved to avoid nuclease contamination

1 RNA dissociation buffer 40 m*M* Tris-HCl, 2 m*M* EDTA, pH 9 0, 2% sodium dodecyl sulfate (SDS) (w/v), 0 5mg/mL bentonite (*see* **Note 6**).
2 TE buffer 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7 5
3 Phenol saturated in TE, pH 7 5 (*see* **Note 1**).
4 Chloroform
5 3*M* Sodium acetate, pH 5 2, with acetic acid (autoclaved)
6 Ethanol (kept at –20°C)

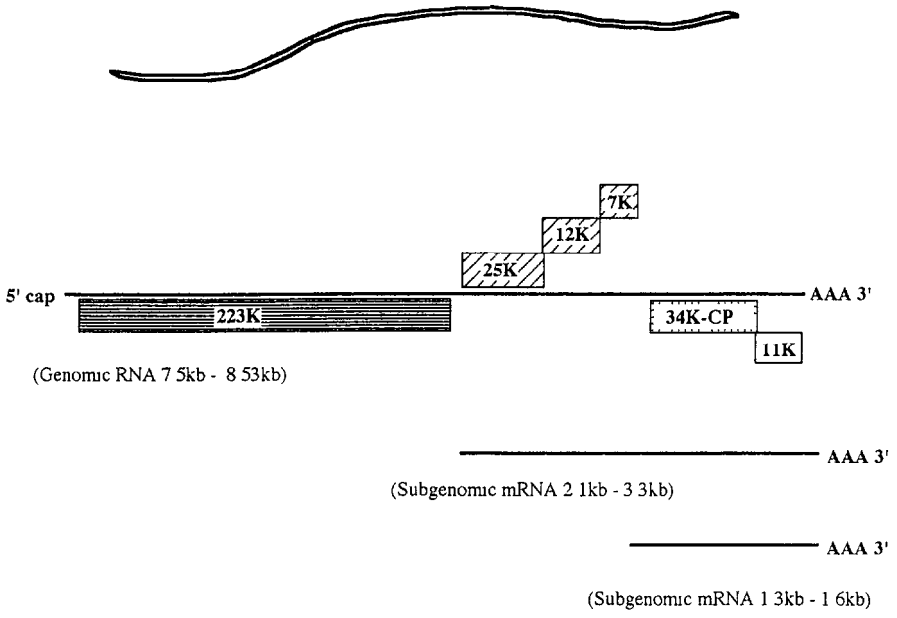



Fig 1 General gene organization of the carlavirus genome Particle morphology, sizes of typical RNAs, and encoded ORFs are indicated

7 Vortexer.
8 Microcentrifuge

## 3. Methods

### *3.1. Virus Isolation*

The method described below has been used by the author to purify a wide range of carlaviruses and has been found to generate high yields of pure virus, when compared with a number of purification procedures for carlaviruses. It should be noted, however, that this purification procedure uses the highly volatile diethyl ether as the clarification agent. If diethyl ether cannot be used for safety reasons, then it is recommended that solvent, such as chloroform, butanol, or carbon tetrachloride should be tried

1. Homogenize infected leaves (*see* **Note 2**) in 0.5*M* borate buffer, pH 7 8, containing EDTA (0 005*M*) and thioglycolic acid (0 1% v/v) at the ratio of 1 2 (w/v)
2 Transfer to a beaker and stir gently with an equal volume of diethyl ether (*see* **Note 3**).
3 Separate phases by centrifugation at 5000*g* for 20 min (at 4°C if possible) and transfer the aqueous phase to a fresh beaker

4. Slowly stir in PEG (6%) (w/v), and continue stirring at 4°C overnight
5. Collect the resulting precipitate by centrifugation at 10,000*g* for 15 min and resuspend in 0.5*M* borate buffer containing Triton X-100 (0.5%) (w/v)
6. Centrifuge at low speed (10,000*g* for 15 min) and transfer the supernatant to a fresh tube.
7. Sediment the virus by high-speed centrifugation (30,000*g* for 90 min) and resuspend the resulting pellet in 0.05*M* borate buffer containing 1% Triton X-100 and sonicate for 20s (21Kc/s) (*see* **Note 4**)
8. Subject the suspension to a further cycle of low- and high-speed centrifugation
9. Purify the virus by isopycnic centrifugation in cesium chloride (0.439 g/mL, 20 h, 5°C, 110,000*g*).
10. Collect the virus band from the cesium gradient (*see* **Note 5**) and dilute at least twofold with 0.005*M* borate buffer
11. Collect the virus by centrifugation (40,000*g* for 90 min) and resuspend the pellet in 0.5 mL of sterile distilled water (*see* **Note 6**)
12. Virus can then be used immediately, stored at 4°C for 1–2 d, or placed at –20°C for long-term storage

### 3.2. RNA Extraction

All of the carlaviruses that we have worked with have yielded good quality RNA from purified particles, using a method essentially as reported by Shields and Wilson *(11)*

To prevent degradation when extracting RNA, gloves should be worn at all times and all tips and microcentrifuge tubes should be autoclaved to avoid nuclease contamination.

1. Add an equal volume of RNA dissociation buffer (*see* **Note 7**) to a volume of virus suspension and mix by vortexing for 5–10 s
2. Incubate at 60°C for 3–5 min.
3. Add an equal volume of phenol, mix briefly, and incubate at 60°C for one more minute
4. Separate phases in a microcentrifuge (12,000*g*) for 2–3 min
5. Remove aqueous phase to a fresh tube and add an equal volume of phenol and an equal volume of chloroform and mix by vortexing for 5–10 s
6. Separate phases in a microcentrifuge (12,000*g*) for 2–3 min
7. Remove aqueous phase to a fresh tube and add an equal volume of chloroform and mix by vortexing for 5–10 sec
8. Separate phases in a microcentrifuge (12,000*g*) for 2–3 min
9. Remove aqueous phase to a fresh tube and precipitate RNA by adding 0.1 vol sodium acetate (3*M*) and 2.5 vol ethanol
10. Store at –20°C overnight, or –70°C for 1 h, before recovering RNA by centrifugation in a microcentrifuge (12,000*g*).
11. Dry the RNA pellet under vacuum to remove all traces of ethanol before resuspending in a suitable volume of sterile distilled water
12. Run a sample of RNA on an agarose to check yield and quality (*see* **Note 8**)

## 4. Notes

1 **Caution:** Care should be taken when working with phenol, because it is highly corrosive Wear gloves at all times and remove all spills immediately.
2 The vast majority of carlaviruses are present in infected tissue at very low concentrations It is therefore recommended to use as much plant material as possible, though this will be dictated by the volume that can be handled during the first two centrifugation steps
3 Add cold diethyl ether, which has been stored in a cold room at 4°C overnight It is also advisable to carry out all work with diethyl ether in a well-ventilated fume hood
4 Carlaviruses will often form aggregates with themselves and with plant material, and we have found sonicating the pellet is the best way to resuspend the virus If a sonicator is not available, the pellet must be resuspended well with a glass rod before further cycles of low- and high-speed centrifugation
5 Virus band can be visualized by shining a light directly at the tube, with the band being removed with a Pasteur pipet or syringe and needle
6 The purity of virus preparation can be checked by comparing the absorbance at wavelengths of 260 and 280 nm in a spectrophotometer Assume an $A_{260}$ of 2 8 for a 1-mg/mL virus preparation Alternatively, view the virus with electron microscopy
7. We have found that the addition of bentonite to the extraction buffer increases the yield of RNA, though it is not essential.
8 Any standard RNA gel technique can be used to analyze RNA quality However, it should be noted that it is typical for carlaviruses to have a band of genomic RNA with a substantial smear of smaller RNAs decreasing in mol wt below it These smaller RNAs are generated from broken particles as part of the virus purification and also as part of the RNA extraction procedure

## References

1 Foster, G D (1992) The structure and expression of the genome of carlaviruses *Res Virol* **143,** 103–112
2 Badge, J , Brunt, A , Carson R., Dagless, E , Karamagioli, M , Phillips, S , Seal, S , Turner, R , and Foster, G D (1996) A carlavirus-specific PCR primer and partial nucleotide sequence provides further evidence for the recognition of cowpea mild mottle virus as a whitefly-transmitted carlavirus *Eur J Plant Pathol* **102,** 305–310.
3 Brunt, A. A. (1995) Carlaviruses, in *Virus Taxonomy Sixth Report of the International Committee on Taxonomy of Viruses* (Murphy, F A , Fauquet, C M , Bishop, H L , Ghabrial, S A , Jarvis, A E , Martelli, G. P , Mayo, M A , and Summers, M D , eds ), Springer-Verlag, Wien, New York
4 Zavriev, S K., Kanyuka, K. V , and Leavy, K. E (1991) The genome organisation of potato virusM RNA *J Gen Virol* **72,** 9–14
5. Cavileer, T. D., Halpern, B T , Lawrence, D. M , Podleckis, E. V , Martin, R. R., and Hillman, B I (1994) Nucleotide sequence of the carlavirus associated with blueberry scorch and similar diseases *J. Gen Virol* **75,** 711–720

6 Foster, G. D and Mills, P. R (1990) Evidence for the role of subgenomic RNA species in the production of Helenium virus S coat protein during in vitro translation *Virus Res* **17,** 61–70
7 Foster, G D and Mills, P R (1992) Translation of potato virus S RNA in vitro. evidence of protein processing *Virus Genes* **6,** 47–52
8 Lawrence, D M , Rozanov, M N , and Hillman, B I (1995) Autocatalytic processing of the 223-kDa protein of blueberry scorch carlavirus by a papain-like proteinase *Virology* **207,** 127–135.
9 Foster, G D. and Mills, P R (1991) Evidence for subgenomic RNAs in leaves infected with an Andean strain of potato virus S *Acta Virologica* **35,** 260–267
10. Foster, G D. and Mills, P R (1990) Evidence for the role of subgenomic RNAs in the production of potato virus S coat protein during in vitro translation *J Gen Virol* **71,** 1247–1249
11 Shields, S A and Wilson, T. M A (1987) Cell free translation of turnip mosaic virus RNA *J Gen Virol* **68,** 169–180

# 14

# Potyvirus Isolation and RNA Extraction

**Philip H. Berger and Patrick J. Shiel**

## 1. Introduction

The *Potyviridae* are the largest single group of plant viruses, and as such are the most important from an economic standpoint. There are nearly 200 distinct recognized species or other viruses that are possible or probable members of the group Two recent books provide an excellent and in depth review of the *Potyviridae (1,2)*. Generally, they are filamentous particles of ca. 11–12 × 680–900 nm. The single-stranded, message sense RNA molecule of the virus genome is encapsidated by single species of CP of ca 30–36 kDa This RNA (ca. 8500–9800 nucleotides) is polyadenylated and also contains a VPg covalently bound to the 5' end. Coat protein amino acid sequences show significant homology, particularly within the core region. Virions are approx 5% RNA and 95% protein by weight. The virus RNA is translated initially as a large polyprotein that is autoproteolytically cleaved to provide the mature viral proteins (**Fig. 1**)

The sheer size and diversity of this group, as well as diversity and range of susceptible hosts, makes description of general procedures difficult, at best. There are probably as many variations on the methods presented below as there are potyviruses. Some of the problems associated with purification are ameliorated by virtue of the ease with which many potyviruses can be manipulated, e.g., mechanical transmission. Although many members of the group have relatively restricted host ranges, most are not so restrictive that suitable propagation hosts cannot be found Therefore, many of the common guidelines outlined in this chapter are applicable in terms of propagation host selection and choice of a purification procedure, if there is no precedent in the literature. Nevertheless, no single method is suitable for purification of all (or even the majority) of potyviruses.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

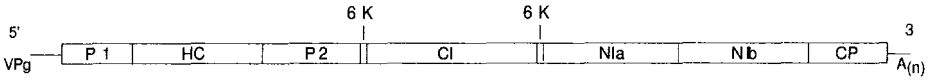



Fig. 1 General gene organization of the potyvirus virus genome

Perhaps the biggest single problem with the purification of potyviruses is aggregation of virions during the purification process. When aggregation occurs, particularly at earlier stages of purification, significant losses occur during the low-speed centrifugation (clarification) steps. Thus, many procedures utilize the nonionic detergent Triton X-100 to prevent this. Once aggregation occurs, it is difficult if not impossible to reverse Here, we present methods that have proven successful for a number of potyviruses Once purified virus is available, methods for purification of viral RNA are relatively straightforward

## 2. Materials

### 2.1. General Materials

1 Organic solvents Reagent grade chloroform ($CHCl_3$) and carbon tetrachloride ($CCl_4$).
2 Other reagents· Polyethylene glycol (PEG) (mol wt 6000 or 8000), Triton X-100, optical grade CsCl, optical grade sucrose, NaCl
3 High capacity (500 mL) centrifuge capable of 10,000*g*
4. Centrifuge tubes resistant to $CHCl_3$ and $CCl_4$ (polypropylene)
5 Ultracentrifuge capable of 120,000*g*
6 Blender. We recommend the use of the 4-L Waring (New Hartford, CT) blender with a stainless steel container. This blender is rather expensive, but it has the torque to handle tough, fibrous tissues. If such a blender is unavailable, be sure to cut coarse, fibrous tissues (like grasses) to 1–2 in or smaller lengths.
7 Stir plate and magnetic stirbars
8 Vortexer
9 40°C Water bath
10 –80°C Freezer

### 2.2. Buffers for General Potyvirus Purification

1 Buffer A 0.5*M* borate, pH 8 0 (boric acid titrated to pH with NaOH)
2 Buffer B· Buffer A with 0 15% sodium thioglycollate (grinding buffer)
3 Buffer C 0 05*M* borate, pH 8 0, with 5 m*M* EDTA

### 2.3. Buffers for Purification of Maize Dwarf Mosaic Virus

1 Buffer A· 0 1*M* ammonium citrate, dibasic, adjusted to pH 6 0 with solid KOH A 1.0*M* stock solution can be prepared

2 Buffer B· Buffer A containing 1% polyvinyl pyrrolidone (mol wt 40,000) and 0 5% 2-mercaptoethanol (grinding buffer).
3 Buffer C Buffer A containing 0.5% 2-mercaptoethanol
4 Buffer D. Buffer C containing 20% (w/v) sucrose
5 Buffer E: Buffer A containing 10, 20, 30, and 40% sucrose (w/v), for preparation of sucrose density gradients

### 2.4. Buffers for Purification of Legume-Infecting Potyviruses

1 Buffer A. 0.5*M* potassium phosphate buffer, pH 7.5.
2 Buffer B: Buffer A containing 0 02*M* sodium sulfite (grinding buffer).
3. Buffer C. 0 25*M* potassium phosphate buffer, pH 7.5 (dilute buffer A 1.1 with deionized $dH_2O$).
4. Buffer D· 20% (w/v) PEG in 0.02*M* Tris-HCl, pH 8 2

### 2.5. Buffers for Purification of Rymoviruses

1 Buffer A Grinding buffer 0 01*M* $K_2HPO_4$ Chill buffer and tissue to 4°C before use
2 Buffer B· 0 01*M* sodium citrate, adjusted to pH 8 0 with 1*M* HCl

### 2.6. RNA Isolation

1. A 10 or 20% sodium dodecyl sulfate (SDS) stock solution made in deionized $dH_2O$
2 Tris-equilibrated phenol, pH 8 0 (available from US Biochem [Cleveland, OH] and other suppliers)
3 Chloroform isoamyl alcohol, 24 1
4 TE buffer· 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
5. 7.5*M* Ammonium acetate
6 Reagent grade ethanol
7 DEPC-treated water: Add diethyl pyrocarbonate to 0 1% and stir vigorously for 1 h. Autoclave for at least 25 min (121°C) Freeze aliquots in RNase-free containers until needed. Care should be taken to avoid contamination with RNase by use of RNase-free (or disposable) containers

## 3. Methods

### 3.1. Potyvirus Purification

This procedure is perhaps the most common starting point when attempting to purify an unknown potyvirus. It was first developed by Moghal and Francki *(3)* and is presented here. Improvements and modifications (from numerous laboratories) are incorporated into the protocol Many of the notes for this method that follow (*see* **Subheading 4.1.**) are applicable or relevant to steps in the other protocols.

1. Grind leaves in 2 vol (400 mL) grinding buffer until thoroughly triturated Add 0.5 vol (v/v) of chloroform and carbon tetrachloride (100 mL each). Blend again for 1 min at highest speed in the blender (*see* **Notes 1** and **2**)

2 Centrifuge at 10,000*g* for 20 min at 4°C
3 Filter aqueous phase through large kimwipes supported by cheesecloth Make sure that no chloroform or carbon tetrachloride remains in this initial extract (*see* **Note 3**)
4 Add PEG (mol wt 8000) to 4% (w/v) and NaCl to 1 75% (w/v) Stir on ice for 1 h (*see* **Note 4**)
5 Centrifuge at 10,000*g* for 15 min at 4°C Carefully discard supernatant and retain the pellets
6 Resuspend pellets in ca 0 1 vol of initial extract, using cold 0 5*M* borate, pH 8 0 (*see* **Note 5**).
7. Just before low-speed centrifugation, add Triton X-100 to 0 25% (v/v) and stir until thoroughly dissolved Low-speed centrifuge at 8000*g* for 15 min at 4°C Retain the supernatant
8 Ultracentrifuge the retained supernatant at 70,000*g* for 1 5 h at 4°C (*see* **Note 6**)
9 Resuspend pellets in buffer C, using about one-thirtieth vol of the initial extract (*see* **Note 5**)
10 Repeat **steps 7–9**, but resuspend pellets in 5 mL buffer C Centrifuge briefly at low speed (8000–10,000*g*) for 1–2 min
11. Layer supernatant on 10–40% sucrose density gradients made in buffer C, and centrifuge 2 h at 95,000*g* in a SW 28 swing bucket rotor (*see* **Notes 7–9**)
12. If desired, dilute virus-containing fractions with at least 3 vol of buffer C and mix thoroughly Ultracentrifuge as in **step 8**, above, to concentrate virus-containing fractions
13 Resuspend pellet in 1 or 2 mL of buffer C (*see* **Note 10**).

### 3.2. Purification of Maize Dwarf Mosaic Virus

This method, originally developed by W. Langenberg, has been used by a number of laboratories for purification of potyviruses infecting Gramineae, particularly maize dwarf mosaic (MDMV) and the related sugarcane mosaic virus (SCMV), sorghum mosaic virus (SrMV), and johnsongrass mosaic virus (JGMV). Langenberg reports that addition of 2*M* guanidine HCl, at **steps 1–6**, will significantly increase virus yield (personal communication).

1 Collect 500–1500 g infected tissue and grind in a large, prechilled blender with a minimum of 1 L buffer B Strain the contents through several layers of cheesecloth, squeezing out as much sap as possible (*see* **Notes 11–13**)
2 Add carbon tetrachloride to 5% (v/v) and mix in a blender for about 5 s
3 Centrifuge for 10 min at 10,000–15,000*g* at 4°C and reserve the supernatant (*see* **Note 14**)
4 While stirring, add Triton X-100 to 0.25% final concentration Then, add solid PEG to 6% final concentration Stir until dissolved (about 0 5 h) (*see* **Notes 15** and **16**)
5. Centrifuge solution for 20 min at 10,000–15,000*g* at 4°C and discard supernatant Resuspend pellet in ca 100 mL buffer C and clarify by centrifuging for 10 min at 10,000*g* at 4°C, reserving the supernatant.

6. Layer the supernatant on a sucrose pad consisting of 5–8 mL buffer D (depending on tube size) and centrifuge 90 min at 100,000*g* at 4°C. Resuspend the pellets in buffer A and centrifuge at 8000*g* at 4°C for 10 min.
7. Layer aliquots of the supernatant on 10–40% sucrose density gradients (buffer E) and centrifuge for 2 h at 100,000*g* Collect the high $A_{254}$ absorbing fraction (*see* **Note 17**)
8. Dialyze the virus-containing fractions overnight against several changes of buffer A, or simply dilute the fractions and centrifuge at high speed to concentrate the virus

### 3.3. Purification of Legume-Infecting Potyviruses

This procedure has been used successfully by several laboratories for purification of strains of bean common mosaic virus, bean common mosaic necrosis virus, and bean yellow mosaic virus. It should be applicable to many other related legume-infecting viruses. The method presented here is a modification of the procedure of Morales, as cited in **ref. *4***.

1. Harvest 200 g leaf tissue 10–11 d after inoculation and homogenize with 200 mL cold grinding buffer (buffer B). Add, for each 200 mL buffer, 50 mL chloroform and 50 mL carbon tetrachloride and homogenize briefly (about 10 s)
2. Centrifuge for 5 min at 5000*g* Pour off supernatant carefully (*see* **Note 18**)
3. Add PEG to 6% (w/v), stir 1 h at 4°C, and centrifuge for 10 min at 12,000*g* (*see* **Note 19**)
4. Allow pellet to resuspend undisturbed in buffer A for at least 6 h, and then clarify by centrifugation for 10 min at 12,000*g* (*see* **Note 20**).
5. Add 2 mL of a 20% (w/v) PEG solution in 0 02*M* Tris-HCl, pH 8 2, per 5 mL virus suspension Incubate 1 h at 4°C.
6. Centrifuge for 10 min at 17,000*g*
7. Resuspend pellets in buffer C You can resuspend this pellet overnight at 4°C
8. Centrifuge for 10 min at 12,000*g*
9. Add CsCl to 35% (w/v), gently dissolve, and centrifuge for 18 h at 120,000*g* (*see* **Note 21**).
10. Recover virus zone Dilute the CsCl with buffer C and concentrate by centrifugation at 84,500*g* for 90 min at 4°C

### 3.4. Purification of Rymoviruses

This method was originally developed by Brakke and Ball *(5)* and subsequently modified by Sherwood *(6)* Although this procedure works quite well, it has the disadvantage that relatively large volumes of extract must be subjected to ultracentrifugation because a PEG precipitation step is not (and cannot be) used. Thus, a large capacity ultracentrifuge rotor is very useful here

1. Grind young leaves (about 3 wk after inoculation) in 2 mL buffer A per gram leaf tissue. Squeeze tissue through cheesecloth as described above (*see* **Notes 22** and **23**).
2. Check pH of filtrate If above pH 6 1, lower to pH 6 1 with acetic acid

3 Incubate filtrate in a 40°C water bath for 1 h (*see* **Note 24**)
4 Centrifuge for 20 min at 10,000*g* at 4°C and retain the supernatant.
5 Adjust supernatant to pH 8 0 with 1*M* NaOH Measure initial extract While stirring, add Triton X-100 to 1.5% (v/v) and sodium citrate powder to 0 01*M* final concentration
6 Centrifuge for 10 min at 10,000*g* at 4°C, and retain the supernatant
7 Centrifuge for 90 min at 100,000*g* at 4°C. Resuspend the pellet in buffer B (*see* **Note 25**)
8 Centrifuge for 10 min at 10,000*g* at 4°C, and retain the supernatant
9 Layer supernatant over 0 2 vol (about 8 mL) 20% sucrose in buffer B and centrifuge at high speed, as in **step 8**
10. Resuspend the pellet in buffer B, and centrifuge briefly at 8000–10,000*g* at 4°C
11 Layer supernatant on a 10–40% sucrose density gradients prepare in buffer B, centrifuge, and collect the virus-containing fraction, as described previously (*see* **Notes 7–9**)
12 Dilute the sucrose-containing fractions with buffer B and centrifuge for 90 min at 100,000*g* at 4°C to concentrate virus Resuspend the pellet in buffer B, which can be resuspended overnight at 4°C (*see* **Note 26**)

### 3.5. RNA Isolation from Virions

1 Virus in the same buffer used after the final purification step can be used here (*see* **Notes 27** and **28**)
2 Add SDS to 1% (v/v) final concentration (i e , add 5 µL of a 20% stock solution for every 100 µL virus preparation)
3 Incubate at 55°C in a heat block or water bath for 5 min
4 Add an equal volume of Tris-equilibrated phenol, preheated to 55°C
5 Vortex vigorously for 5 s and centrifuge at 10,000*g* for 2 min
6. Remove aqueous phase to a fresh (RNase-free) microcentrifuge tube Add an equal volume of chloroform (chloroform:isoamyl alcohol [24·1])
7 Vortex vigorously for 5 s and centrifuge at 10,000*g* for 1 min
8 Remove aqueous phase to a fresh (RNase-free) microcentrifuge tube Add 0 5 vol of 7 5*M* ammonium acetate. To this, add 2 5 vol of cold (–20°C) reagent grade ethanol
9 Invert tubes until fully mixed. Place in the cold (–80°C for 30 min or –20°C for 1 h)
10. Centrifuge at 12,000*g* for 25 min at 4°C Remove supernatant and save pellet Add 1 vol (of original virus prep) of cold 70% ethanol (made with DEPC-treated water).
11 Immediately centrifuge at 12,000*g* for 5 min Remove supernatant and save pellet Vacuum-dry pellet (10–15 min without heat)
12 Resuspend in a small volume (ca 20 µL of DEPC-treated water or RNase-free TE buffer).
13 Dilute a 5-µL aliquot in DEPC-treated water to obtain absorbances at 260 and 280 nm on an UV spectrophotometer to determine yield (20 $OD_{260}$ = 1 mg RNA)

In addition, you can electrophorese 5 µL RNA (mixed with 3 µL of an RNase free loading dye) on a 2% agarose gel in TAE buffer (Tris-acetate-EDTA) to obtain quality and yield information Potyviral RNA should migrate as a clear single band with little or no degradation Store RNA preparations at –80°C (*see* **Notes 29** and **30**)

## 4. Notes

### *4.1. Potyvirus Purification*

1 This procedure is designed for 200 g of infected tissue, but can be scaled-up by concomitant increase in added reagents When working with virus hosts that result in viscous extracts (e g , hosts in the Malvaceae or *Allium* spp.), try including 2% (w/v) Polyclar AT (Sigma, St. Louis, MO) and 0 02*M* sodium sulfite to the extraction buffer Many procedures also will use EDTA (0 05*M*) in the extraction buffer The need for these or other amendments can only be determined empirically

2 (**Subheading 3.1., step 1**) Excessive mixing may cause foaming A foam suppresser, available for the 4 L Waring blender, will reduce this **Caution:** Protective clothing and gloves should be used when handling organic reagents Chloroform and carbon tetrachloride cause burning pain and redness if contact with skin occurs Inhalation or ingestion can cause central nervous system depression, with dizziness, drowsiness, and vomiting These reagents are suspected carcinogens based on animal studies

3. (**Subheading 3.1., step 3**) Use a separatory funnel, if necessary, to remove all carbon tetrachloride and chloroform

4 (**Subheading 3.1., step 4**) A common source of virus loss is failure to release virus from the PEG In many procedures, NaCl is included to aid in virus release and, although not used in some procedures, it may be important Therefore, it may be useful to consider inclusion of 1 5–1 75% (w/v) NaCl when purifying an uncharacterized virus, if yields are unsatisfactory

5. (**Subheading 3.1., steps 6** and **9**) Do not resuspend using vortex mixer You can resuspend overnight at 4°C, if you wish

6. (**Subheading 3.1., step 8**) This is about 30,000 rpm in a Beckman Ti 70 rotor

7 (**Subheading 3.1., step 11**) It is important not to add more than 2 mL (derived from the equivalent of 50–75 g tissue) to every 35 mL sucrose gradient Overloading by using more than 2 mg virus per gradient will likely cause virion aggregation and precipitation Prevention of this can be enhanced by the addition of Triton X-100 to the sucrose solutions (to 0.1% final concentration) before gradient preparation However, Triton X-100 strongly absorbs light at 254 nm and can make fractionation using a spectrophotometer (i e., ISCO system) difficult

8. (**Subheading 3.1., step 11**) Assumption here is that a Beckman SW 28 rotor or equivalent is used Centrifugation times will have to be adjusted if a different rotor is used Vertical or fixed-angle rotors can be used for sucrose density gradi-

ent centrifugation, but separation may be unsatisfactory if there is turbulence during deceleration caused by even slight rotor imbalance

9 (**Subheading 3.1., step 11**) Visualize virus-containing band after centrifugation by shining a light (like from a binocular microscope light source) You can remove this band directly with a Pasteur pipet, or fractionate it using absorbed light at 254 nm with a commercial fractionator

10 (**Subheading 3.1., step 13**) We recommend that one determine absorbance, regardless of purification method used, at 260 and 280 nm spectrophotometrically The 260/280 ratio for potyviruses should be around 1 2–1 3 (or up to 1 4 for rymoviruses) Readings that are well out of this range (more than ca 0 1 U) indicate probable contamination and/or degraded virus Store potyviruses by refrigeration at 4°C in the presence of 0 04% (w/v) $NaN_3$. EDTA can also be added as a preservative We do not recommend freezing virus preparations and, although there appears to be wide variation in storage life of potyviruses, most should remain stable and useful for several months

## 4.2. MDMV Purification

11 Titer of viruses such as MDMV does not seem to be as time-dependent as many dicot-infecting viruses, but yields will be greater if younger infected tissue is used, rather than older leaf tissue. Some workers remove leaf mid-rib tissue prior to grinding

12 (**Subheading 3.2., step 1**) Try to keep the ratio of extraction buffer to tissue low, in the 1·1–1·1 5 range, if possible

13. (**Subheading 3.2., step 1**) Perhaps the greatest loss of virus at this stage is caused by poor or incomplete grinding of tissue and failure to extract as much sap as possible from the cheesecloth

14 (**Subheading 3.2., step 3**) Be sure to carefully decant the supernatant to remove all carbon tetrachloride Failure to do so will require repeating **Subheading 3.2., step 3**

15 (**Subheading 3.2., step 4**) Making a 25% stock solution will make it easier to pipet Triton X-100

16. (**Subheading 3.2., step 4**) Do not stop at this point Extended contact between virus and the detergent will significantly reduce yield

17 (**Subheading 3.2., step 7**) The band containing virus should be clearly visible if the tube is illuminated from the bottom Alternatively, one can simply remove the virus-containing fraction by pipeting gently with a U-shaped Pasteur pipet, or by inserting a 20-gage needle through the side of the tube Most laboratories will use an ISCO density gradient fractionation system to retrieve the virus-containing band

## 4.3. Purification of Legume-Infecting Potyviruses

18 (**Subheading 3.3., step 2**) The organic phase will come loose, but you do not want any of this mixed with supernatant. Pour off the rest into a separatory fun-

nel, allow to separate, and add the remainder of aqueous phase to rest of supernatant Discard pellet and organic solvents in proper containers

19 (**Subheading 3.3., step 3**) Alternatively, add PEG to 4% (w/v) and NaCl to 0.25*M* We have done this both ways and occasionally some viruses or virus strains appear to yield better with this method, rather than with 6% PEG and no NaCl

20 (**Subheading 3.3., step 4**) We have done this for less time with no apparent loss in yield. Probably as little as 2 h is adequate.

21. (**Subheading 3.3., step 9**) One may use thick-wall polycarbonate tubes and the Ti 70 rotor at 125,000*g* Fill these tubes no more than half full If your ultracentrifuge will calculate $\omega^2 t$, use ≈12,000. Some potyviruses are unstable in CsCl This will be apparent if most of the virus is lost during ultracentrifugation to remove the CsCl If this should happen, use 35% (w/v) $Cs_2SO_4$, rather than CsCl.

## 4.4. Purification of Rymoviruses

22 (**Subheading 3.4., step 1**) Brakke and Ball *(2)* report that the best yields are obtained from younger leaves, but not until these leaves had well-developed symptoms throughout.

23 (**Subheading 3.4., step 1**) Take care not to heat up blender excessively

24 (**Subheading 3.4., step 3**) It is best to keep track of temperature during this period of time

25. (**Subheading 3.4., step 7**) When resuspending the first high-speed pellet, use about one-thirtieth vol of the initial extract This step can be performed overnight at 4°C.

26 (**Subheading 3.4., step 12**) Our average yield for WSMV is about 1 2 $A_{254}$ units, or about 0 4 mg/100 g tissue, using 3 0 as the extinction coefficient The $A_{260/280}$ should be ≈1 37.

## 4.5. RNA Isolation from Virions

27 Do not use buffers that have over 50 m*M* potassium in them **Caution:** Do not inhale SDS and avoid contact with skin, eyes, and clothing. Phenol is toxic if in contact with skin or swallowed and causes burns Always use with gloves and protective eye wear DEPC is toxic if in contact with skin or swallowed and causes burns Always use with gloves and protective eye wear

28 Use virus at concentrations greater than 250 µg/mL whenever possible Lower starting concentrations of virus will adversely affect RNA yields.

29 Nucleic acid content of potyviruses is about 5%. One milligram of virus should yield close to 50 µg of RNA.

30. Although isolation of viral RNA is necessary for some experimental procedures involving viruses, it is not always necessary to isolate viral RNA to clone viruses via reverse transcription Recent work has shown that a simple procedure can be used to obtain high-quality cDNA directly from potyviral virions *(7)* This procedure circumvents the sometimes difficult isolation of high-quality, pure RNA from virions Full-length or near-full-length cDNA from potyviruses can be syn-

thesized using this method and can be incorporated into an immunocapture/reverse transcriptase/polymerase chain reaction procedure (S D Wyatt, personal communication)

## References


1 Barnett, O W (1992) *Potyvirus Taxonomy* Springer-Verlag, Vienna
2. Shukla, D D , Ward, C W , and Brunt, A A (1994) *The Potyviridae* CAB International, Wallingford, UK
3 Moghal, S M and Francki, R I. B. (1976) Towards a system for the identification and classification of potyviruses. 1 Serology and amino acid composition of six distinct viruses *Virology* **73,** 350–362
4. Morales, F. J and Bos, L (1988) Bean common mosaic virus, no 337, in *AAB Descriptions of Plant Viruses* (Murant, A F and Harrison, B D , eds ), Association of Applied Biologists, Institute of Horticultural Research, Wellesbourne, UK
5 Brakke, M K and Ball, E. M (1968) Purification and antigenicity of wheat streak mosaic virus *Phytopathology* **58,** 963–971.
6 Sherwood, J. L (1987) Comparison of a filter paper immunobinding assay, western blotting and an enzyme linked immunosorbent assay for the detection of wheat streak mosaic virus *J Phytopathol* **118,** 68–75
7 Wyatt, S D , Druffel, K , and Berger, P H (1993) *In vitro* destabilization of plant viruses and cDNA synthesis. *J Virol Methods* **44,** 211–220

# 15

# Trichovirus Isolation and RNA Extraction

**Sylvie German-Retana, Thierry Candresse, and Jean Dunez**

## 1. Introduction

### *1.1. Trichoviruses: A New Plant Virus Genus*

The genus Trichovirus, a newly established plant virus genus *(1)*, contains five viral species (including three tentative members), with similar biological, morphological, physicochemical, and ultrastructural properties. Apple chlorotic leaf spot virus (ACLSV) and potato virus T (PVT) are definitive members of the genus *(2,3)*, whereas *heracleum* latent virus (HLV) *(4)*, grapevine virus A (GVA), and grapevine virus B (GVB) *(5,6)* are regarded as putative members.

ACLSV, the type-member of the Trichovirus genus, has previously been classified in the closterovirus group, according to the morphology of its flexuous and filamentous viral particle *(7)*. ACLSV was the first clostero-like virus whose complete genome was sequenced and genomic organization determined *(8,9)*. When molecular information became available on other closteroviruses *(10,11)*, it became evident that there were differences in genome properties and structure between ACLSV and beet yellows virus (BYV), the type-member of the closterovirus group. Those molecular differences, when added to the differences in particle and genome length, vector transmission, and cytopathic inclusions, led to the establishment of a new viral genus called Trichovirus ("tricho" from the Greek "thrix," meaning hair), which was approved by the ICTV committee at the Ninth International Congress of Virology, Glasgow, 1993.

Most of the individual species of the trichovirus genus are fairly well-characterized biologically and physicochemically.

Complete sequence data is available for two strains of ACLSV *(8,12)*; whereas only partial sequences are available for PVT, GVA, and GVB *(13,14)*, and none for HLV.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

## 1.2. Biological Properties

### 1.2.1. Host Range

Trichoviruses infect dicotyledonous plants and differ in their geographical distribution. ACLSV, GVA, and GVB are found worldwide, but PVT was reported only in the Andean region of South America. The natural host range of trichoviruses is restricted to either a single host (PVT, HLV, GVA, and GVB), or to a somewhat wider host range (ACLSV).

ACLSV is known to infect woody rosaceous plants, including apple, pear, plum, peach, cherry, and apricot. Although it is more or less symptomless in pome fruits, it is responsible for serious diseases in stone fruits, including peach dark-green mottle, false plum pox, and plum bark split *(15)*. The economic importance of ACLSV is largely because of its worldwide distribution, and to its capacity to induce severe graft incompatibilities in some *Prunus* combinations, which causes important problems in nurseries.

PVT host range is limited: The main disease has been reported only for potato (*Solanum tuberosum*), in which it is usually latent, but occasionally produces a mild leaf mottle *(3)* GVA and GVB are associated with, respectively, Kober stem grooving and corky bark diseases of the grapevine–rugose wood complex. These diseases are associated with symptoms of pitting and grooving *(5,6)* HLV occurs commonly in Scotland in wild *Heracleum sphondylium* (hogweed) plants, without causing any symptoms *(4)*

### 1.2.2 Transmission

All trichoviruses are experimentally transmitted by sap inoculation and by grafting. The viruses can be propagated on the following herbaceous hosts: *Chenopodium quinoa* (ACLSV, PVT, and HLV), *Nicotiana benthamiana* (GVA), and *Nicotiana occidentalis* (GVB). The mode of natural transmission differs among the species. No natural vectors are known for ACLSV and PVT, dissemination being mediated by propagative material, and PVT is also seed transmitted in different hosts. Seed transmission also seems to be possible for ACLSV in apricot *(15)*. Both GVA and GVB are transmitted by mealybugs. HLV is transmitted by aphids in a semipersistant mode.

### 1.2.3. Morphological and Physicochemical Properties

The morphology of all trichoviruses is characterized by a highly flexuous filamentous particle of 12 nm in diameter and 640–800 nm in length, with a pitch of 3.3–3.5 nm and approx 10 subunits per turn of the helix. The viral particle encapsidates a single-stranded positive sense genomic RNA of 2 2–2 5

$\times 10^6$ Dalton (about 7.5 kb) that accounts for 5% of the particle weight. The particles contain a single coat protein (CP) species with a mol wt of 22–27 kDa.

### 1.3. Molecular Properties

The genomic RNA of ACLSV is polyadenylated *(16)* and is probably capped at its 5' end *(8)*. The determination of the complete or partial nucleotide sequence of the RNA of some members of the group *(8,12–14)* has provided knowledge on the genome organization and the gene expression strategy of trichoviruses *(9)*. The ACLSV RNA contains at least three open reading frames (ORFs), encoding proteins with approximate mol wt of 216, 50, and 22 kDa (**Fig. 1**).

The 5' large ORF1 codes for a protein that contains three signature sequences, typical of replicase-associated proteins of the α-like supergroup of plant viruses: The methyl-transferase, nucleotide-binding site helicase, and polymerase signatures *(8)*

The ORF2 of ACLSV shares distant similarities with the cauliflower mosaic virus gene I and TMV 30-kDa movement proteins (MP) *(8)* The ORF2 encoded protein has been included in the proposed family I of MP *(17)*, which also includes the MP of tobamoviruses, tobraviruses, comoviruses, caulimoviruses, and geminiviruses.

The capsid protein ORF is located at the 3' terminus of the genomic RNA. The CP contains motifs that are present in a highly conserved region of filamentous virus CPs, hypothesized to be involved in the formation of a salt bridge, and possibly vital for tertiary structure formation *(18)*.

As shown in **Fig. 1**, GVA and GVB have an additional small ORF at the 3' end of the genome, downstream of the CP gene, which is missing in ACLSV and PVT *(14)*. In the case of GVB, this small ORF has homologies with the small, cystein rich, nucleic acid-binding proteins found in the genome of other plant viruses, such as hordeiviruses and carlaviruses. Distinct sequence homologies exist between the movement proteins and CPs of all members of the trichovirus group.

### 1.4. Virus Purification

The trichovirus group includes viruses that are routinely propagated in different herbaceous hosts, so that different protocols for virus purification are used for the various members. This chapter describes in detail the method of purification currently used in our laboratory for ACLSV, the type member of the trichoviruses. The reader is directed to the references for sources on other trichoviruses.

ACLSV purification is done by the bentonite–polyethylene glycol procedure adapted from Lister and Hadidi *(19)*, with modifications by Dunez et al. *(20)* and other unpublished modifications.

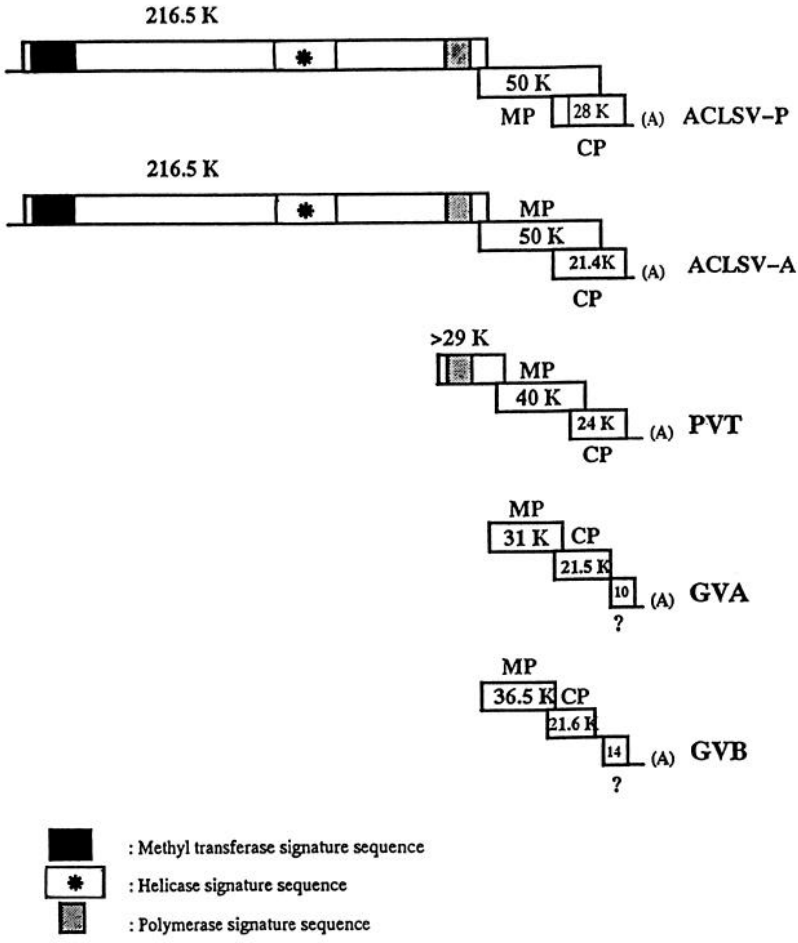



Fig. 1. Comparison of the genomic organization of the complete ACLSV RNA (strain "P" isolated from *Prunus sp.*, and strain "A" isolated from *Malus sp.*), with the 3'-terminal regions of PVT, GVA, and GVB RNAs. The position of the ORFs (boxed) and the sizes of their putative translation products are shown. MP, putative movement protein; CP, coat protein.

## 2. Materials

### 2.1. Virus Purification

1. Buffer A: 10 m*M* Tris-HCl, pH 7.5 (autoclaved) (Bioprobe Systems).
2. Buffer B: 10 m*M* Tris-HCl, 5 m*M* $MgCl_2$ (Prolab), 0.2% 3–3' diaminodipropylamine (Fluka Chemika), pH 7.8. This buffer should be freshly prepared and the pH should be adjusted after the addition of the polyamines.

3 Buffer C 10 m*M* Tris-HCl, 5 m*M* $MgCl_2$, pH 7 8 (autoclaved)
4. 10% Sucrose: 10 g of sucrose dissolved in 100 mL of buffer C
5 40% Sucrose 40 g of sucrose dissolved in 100 mL of buffer C

The last two solutions are used to prepare sucrose gradients and should be kept aliquoted and frozen to avoid bacterial contamination.

### 2.2. Viral RNA Extraction

1 Proteinase K (20 mg/mL in water) (Sigma)
2 20% SDS (w/v) (Bioprobe)
3. Phenol saturated with 50 m*M* Tris-HCl, pH 8 0 (Bioprobe)
4. Chloroform/isoamyl alcohol (24/1, v/v) (Prolabo)
5 3*M* Sodium acetate, pH 5 3 (autoclaved) (Sigma)
6 96% Ethanol (kept at –20°C) (Carlo Erba)
7 TE. 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 (autoclaved)

### 2.3. Gel Electrophoresis of RNA

1 10X MOPS/EDTA, pH 7 0 500 m*M* MOPS (Sigma), 10 m*M* EDTA, adjust to pH 7 0 with NaOH (Sigma) (autoclaving of this buffer will result in a yellow color that does not interfere with the migration)
2 Buffer D 294 µL 10X MOPS/EDTA, pH 7 0, 706 µL $H_2O$
3 Buffer E· 89 µL formaldehyde (Sigma) (37%, 12 3*M*), 250 µL formamide (Sigma) (freshly deionized)
4 Dyes mix 322 µL buffer D, 5 mg xylene cyanol (Serva), 5 mg bromocresol green (Sigma), and 400 mg sucrose
5 Gel-loading buffer· 2 µL formaldehyde (37%, 12 3*M*), 5 µL formamide (freshly deionized), and 7 µL Dyes mix
6 Electrophoresis buffer 1X MOPS/EDTA, pH 7 0
7 Gel preparation The 0 8% agarose gel is prepared by dissolving 0 4 g agarose (Eurobio) in 36 mL of $H_2O$, cooling to 70°C, and adding 5 mL of 10X MOPS/EDTA, pH 7 0, and 9 mL 37% formaldehyde (final concentration 2 2*M*)
8 Gel-staining solution 10 m*M* sodium acetate (Merck), 10 m*M* magnesium acetate (Merck), 0 05% (w/v) Ortho-toluidine blue (Sigma), pH adjusted to 5 5 with acetic acid

## 3. Methods

The following procedure was adopted for purifying ACLSV. All steps are done at 4°C on a refrigerated bench

### 3.1. Bentonite Suspension Preparation

The protocol we currently use in our laboratory is the one proposed by Lister and Hadidi *(19)*, with a few modifications. For a typical batch, 10 g of bentonite (Bentonite powder, Fisher Scientific) are suspended in 200 mL of buffer A

by blending in an electric blender. The bentonite fraction, which does not pellet in 3 min at 600*g* (first centrifugation), but which does pellet in 15 min at 5500*g* (second centrifugation), is resuspended in 100 mL of buffer and kept overnight at 4°C. The following day, the same procedure is repeated, but the last pellet obtained is finally resuspended in 50 mL of buffer A using a blender, resulting in a suspension ready to use, and which contains about 40–50 mg/mL of bentonite. It is important to correctly estimate the bentonite titer by weighing 1 mL of the bentonite suspension after water evaporation.

## 3.2. Virus Purification

### 3.2.1 Grinding

ACLSV is propagated in the herbaceous host *Chenopodium quinoa* (*see* **Note 1**). Symptoms vary depending on the ACLSV strain. Sunken or necrotic lesions can develop after 4–6 d on inoculated leaves, followed by systemic yellow spotting or mottling 2 d later on noninoculated apical leaves.

ACLSV is purified from systemically infected leaves harvested 7–10 d after inoculation Leaves can be kept frozen before purification, but should not be kept longer than 2 mo at –20°C. Leaves (100 g) are homogenized in 3 vol of buffer B in a blender. Both $Mg^{2+}$ ions and polyamines tend to limit viral particle degradation during the purification process

### 3.2.2. Clarification

The homogenate is strained through cheesecloth and then clarified by adding bentonite suspension in steps, starting with an initial amount of 10 mg of bentonite per gram of leaves. The homogenate is mixed, kept at 4°C for 10 min, and then centrifuged 5 min at 1400*g* The supernatant is recovered, and bentonite is added again to a final concentration of 5 mg/g of leaves. The homogenate is mixed and kept at 4°C for 10 min, followed by another centrifugation at 1400*g* for 5 min. This step is repeated until the supernatant becomes straw yellow in color and the pellet grayish, each time using decreasing amounts of bentonite (2.5 mg/g, 1.25 mg/g, and so on).

Bentonite is used to adsorb plant material (organelles, membranes, and so on), which is then pelleted and eliminated after centrifugation. It is important to keep in mind that an excessive use of bentonite will lead to the adsorption of virus particles, and loss of virus. Therefore, the bentonite clarification procedure must be performed very carefully and should generally not exceed four successive steps.

### 3.2.3. Polyethylene Glycol (PEG) Precipitation

The virus is precipitated from the bentonite-clarified extract by adding PEG (mol wt 6000, Merck) to 8% (w/v) of the volume of the clarified extract (*see*

**Note 2**). The solution is gently stirred until the PEG is completely dissolved, then held without stirring for 1 h at 4°C. After centrifugation for 30 min at 12,000*g*, the pellets are resuspended in a maximum of 3 mL of buffer C (*see* **Note 3**).

### *3.2.4. Separation on Sucrose Gradient*

Sucrose gradients 10–40% are prepared from two stock sucrose solutions (*see* **Subheading 2.1., items 4** and **5**). Viral pellets, resuspended in buffer C, are loaded directly onto six gradients (500 µL per gradient) and ultracentrifuged for 2.5 h at 300,000*g*, in a Beckman SW 41 rotor. Gradient tubes are scanned at 254 nm with a ISCO ultraviolet absorption monitor and the UV absorbing regions corresponding to virus fractions are collected in ultracentrifuge tubes.

### *3.2 5. Concentration*

To concentrate the virus, the virus-containing fractions are ultracentrifuged 16 h at 95,000*g* in a Beckman 60 Ti rotor. The final virus pellet is then resuspended in 200 µL of buffer C. The virus concentration can be estimated by measuring the absorption at 254 nm. Concentration is then calculated based on the formula: 2 4 U of $OD_{254nm}$ correspond to a concentration of 1 mg/mL of virus

## *3.3. Viral RNA Isolation*

### *3.3.1. RNA Extraction*

The virus suspension is incubated in the presence of proteinase K (200 ng/µL) and 0.5% SDS for 15 min at 50°C. This suspension is then extracted with an equal volume of Tris-HCl saturated-phenol:chloroform:isoamyl alcohol (25:24:1, [v/v/v]), and then with an equal volume of chloroform:isoamyl alcohol (24:1, [v/v]). The aqueous phase is ethanol-precipitated in the presence of 0.1 vol of 3*M* sodium acetate, pH 5.3, and 2.5 vol of 96% ethanol at –20°C, for at least 1 h. Viral RNA is recovered by centrifugation for 20 min at 4°C at 15,000*g*. The RNA pellet is finally resuspended in 10–20 µL of TE buffer or water

### *3.3.2. Analysis of Viral Nucleic Acid*

RNA is denatured with formaldehyde and electrophoresed in a 0.8% agarose formaldehyde gel, as described by Miller ***(21)***, with few modifications (*see* **Note 4**) The RNA volume should be reduced to 1 µL, to which 2 2 µL of buffer D and 5.8 µL of buffer E (*see* **Subheading 2.3., item 3**) are added. After denaturation by heating at 70°C for 10 min, 1 µL of gel-loading buffer is added and the sample is loaded on the gel (*see* **Subheading 2.3., items 5–7**). After electrophoresis, the gel is stained for 15 min and destained in water (*see* **Subheading 2.3., item 8**)

### 3.4. Yields of Virus and Viral RNA

ACLSV is known to replicate at a relatively low level in the infected plants. Yields of virus typically range from 100 µg to 400 µg/100 g tissue. This corresponds to about 5–20 µg of viral RNA/100 g tissue. This yield can vary, depending on the experiment, but rarely exceeds 20 µg. In the case of the Japanese apple strain of ACLSV, the reported yield was higher: 50 µg of viral RNA/100 g tissue *(12)*. Similar virus yields are obtained for PVT. 300 µg to 1 mg of virus/100 g tissue, corresponding to 15 µg to 50 µg of RNA *(3)*.

The purification yield is reportedly higher for HLV: Yields of virus average 5 mg/100 g tissue, which is about 250 µg of viral RNA *(4)*.

### 3.5. Purification of Other Trichoviruses

GVB is purified from leaves of *N. occidentalis* The method also includes clarification by bentonite, followed by differential centrifugation and sucrose density gradient centrifugation *(6)*. GVA is purified from leaves of *N clevelandii*. The method includes either clarification by bentonite or chloroform extraction, before PEG precipitation *(5)*.

A protocol very similar to the one given for ACLSV is used for HLV *(4)*

For PVT, the method does not include the bentonite clarification step, but uses carbon tetrachloride clarification instead *(13)*.

## 4. Notes

1. Inoculation of *C quinoa* (young plants at the four-node stage) (*see* **Subheading 3.2., step 1**) is done by dusting leaves with Carborundum (600-mesh), then rubbing pairs of leaves at the third node with infectious *C quinoa* sap This sap is prepared by grinding 10 g of infected leaves of *C quinoa* in 30 mL of buffer B, and by adding activated coal (90 mg/mL) in this homogenate just before the inoculation The infectious *C. quinoa* sap must be kept at 4°C It must be noted that ACLSV is very susceptible to high temperatures, therefore, inoculation should not be done during the summer period Grinding the leaves in the blender should be done carefuly and should not last too long, because excessive blending will result in virus degradation. The homogenate obtained should be thick and not too liquid The choice of the Fisher Scientific brand to prepare the bentonite solution seems to make a difference in the purification issue
2. The choice of the Merck brand for PEG also seems to make a difference for the effective viral particles precipitation (*see* **Subheading 3.2., step 3**)
3. The virus pellet after PEG precipitation (*see* **Subheading 3.2., step 3**) may be contaminated by plant components and appear very green Because excess bentonite can lead to the loss of virus particles, it is better not to exceed four steps of clarification To remove any noticeable green color from the pellet, additional differential centrifugation steps can be done before loading onto the gradients, to avoid interference in the UV absorption analysis of the gradients The virus pellet

after PEG precipitation is resuspended in 10 mL of buffer C, and then subjected to centrifugation for 10 min at 10,000*g* The first supernatant is collected, and the pellet is washed again with 10 mL of buffer C After centrifugation the supernatants are pooled and subjected to high-speed centrifugation for 2 h at 160,000*g* in a Beckman 60 Ti rotor The final virus pellet is then resuspended in 3 mL of buffer C and loaded onto the sucrose gradients (*see* **Subheading 3.2., step 4**)

4. The method we present for gel analysis of the viral RNA can be substituted by any other molecular biology technique of nucleic acid analysis described by Sambrook et al *(23)*

## References


1 Martelli, G P, Candresse, T, and Namba, S. (1994) Trichovirus, a new genus of plant viruses, *Arch Virol* **134,** 451–455

2. Lister, R. M (1970) Apple chlorotic leaf spot virus Commonwealth Mycological Institute/Association of Applied Biologists, Description of plant viruses, n° 30

3. Salazar, L. F. and Harrison, B D (1978) Potato virus T Commonwealth Mycological Institute/Association of Applied Biologists, Description of plant viruses, n° 187

4 Bem, F and Murant, A F (1979) Host range, purification and serological properties of heracleum latent virus *Ann Appl Biol* **92,** 243–256

5 Conti, M , Milne, R G , Luisoni, E , and Boccardo, G (1980) A closterovirus from a stem-pitting diseased grapevine *Phytopathology* **70,** 394–399

6 Boscia, D , Savino, V , Minafra, A , Namba, S , Elicio, V , Castellano, M A , Gonsalves, D , and Martelli, G P (1993) Properties of a filamentous virus isolated from grapevines affected by corky bark *Arch Virol* **130,** 109–120.

7. Bar-Joseph, M and Murant, A F (1979) Closterovirus group Commonwealth Mycological Institute/Association of Applied Biologists, Description of plant viruses, n° 260

8. German, S., Candresse, T , Lanneau, M., Huet, J C., Pernollet, J C , and Dunez, J (1990) Nucleotide sequence and genomic organization of apple chlorotic leaf spot closterovirus *Virology* **179,** 104–112

9 German, S , Candresse, T , Le Gall, O , Lanneau, M , and Dunez, J. (1992) Analysis of dsRNAs of apple chlorotic leaf spot virus. *J Gen Virol* **73,** 767–773

10 Agranovsky, A. A., Koonin, E V , Boyko, V P , Maiss, E , Frotschl, R , Lunina, N A., and Atabekov, J G (1994) Beet Yellows Closterovirus complete genome structure and identification of a leader papain-like thiol protease *Virology* **198,** 311–324

11. Pappu, H. R., Karasev, A. V., Anderson, E. J., Pappu, S. S., Hilf, M. E., Febres, V J , Eckloff, R M G , McCaffery, M , Boyko, V , Gowda, S , Dolja, V V , Koonin, E. V , Gumpf, D J , Cline, K C , Garnsey, S M., Dawson, W O , Lee, R F , and Niblett, C L (1994) Nucleotide sequence and organization of eight 3' open reading frames of the Citrus Tristeza closterovirus genome *Virology* **199,** 35–46

12 Sato, K , Yoshikawa, N , and Takahashi, T (1993) Complete nucleotide sequence of the genome of an apple isolate of apple chlorotic leaf spot virus *J Gen Virol* **74,** 1927–1931

13 Ochi, M , Kashiwazaki, S , Hiratsuka, K , Namba, S , and Tsuchizaki, T (1992) Nucleotide sequence of the 3'-terminal region of potato virus T RNA *Ann Phytopathol Soc Japan* **58,** 416–425
14. Minafra, A , Saldarelli, P , Grieco, F , and Martelli, G P (1994) Nucleotide sequence of the 3' terminal region of the RNA of two filamentous grapevine viruses *Arch Virol* **137,** 249–261
15 Dunez, J and Delbos, R P (1988) Closteroviruses, in *European Handbook of Plant Diseases* (Smith, I M , Dunez, J , Lelliott, R A , Phillips, D H , and Archer, S A , eds ), Blackwell, Oxford, pp 5–7
16 Yoshikawa, N and Takahashi, T (1988) Properties of RNAs and proteins of apple stem grooving and apple chlorotic leaf spot viruses *J Gen Virol* **69,** 241–245
17 Koonin, E V, Mushegian, A R , Ryabov, E V, and Dolja, V V (1991) Diverse groups of plant RNA and DNA viruses share related movement proteins that may possess chaperone-like activity *J Gen Virol* **72,** 2895–2903
18. Dolja, V A., Boyka, V P, Agranovsky, A A , and Koonin, E V. (1991) Phylogeny of capsid proteins of rod-shaped and filamentous RNA plant viruses two families with distinct patterns of sequence and probably structure conservation *Virology* **184,** 79–86
19 Lister, R M and Hadidi, A F (1971) Some properties of apple chlorotic leaf spot virus and their relation to purification problems *Virology* **45,** 240–251
20 Dunez, J , Delbos, R., and Bertranet (1973) Rôle de différents facteurs de stabilisation appliqués à la purification du virus du chlorotic leaf spot du pommier. *Ann Phytopathol* **5(3),** 255–264
21 Miller, K (1987) Gel electrophoresis of RNA *Focus* **9,** 3
22 Salazar, L F and Harrison, B D (1978) Host range, purification and properties of potato virus T *Ann Appl Biol* **89,** 223–235
23 Sambrook, J , Fritsh, E F , and Maniatis, T (1989) *Molecular Cloning A Laboratory Manual,* 2nd ed Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

# 16

# Ilarvirus Isolation and RNA Extraction

**Deyin Guo, Edgar Maiss, and Günter Adam**

## 1. Introduction

The genus *Ilarvirus* belongs to the family *Bromoviridae*, together with three other genera *Bromovirus, Cucumovirus,* and *Alfamovirus* ***(1)*** The ilarvirus genus includes at present 15 approved species and is divided into 10 subgroups according to serological relationships ***(2)***. The members are listed in **Table 1**.

Most of the members of the ilarvirus genus have a wide host range and infect woody plants. They can cause diseases of economical importance in stone fruit trees (*Prunus* spp.), apple, hop, citrus, and rose plants ***(2,3)*** The type member, tobacco streak virus (TSV), however, infects mainly herbaceous plants and causes diseases in tobacco, dahlia, cotton, tomato, asparagus, and some legume species The chief measure to control ilarviruses is the use of virus-free propagating material, but the healthy plants can become reinfected easily, since many ilarviruses are transmitted by pollen. Engineered resistance may become a promising perspective for the future control of ilarviruses ***(4)***.

The morphology of ilarvirus particles is quasi-isometric, with diameters between 23 and 35 nm. Occasionally, bacilliform particles are visible in the electron microscope (EM) (**Fig. 1A**) with 12–35 nm width and 20–38 nm length ***(2,3)***. The variability in particle size is caused by the encapsidation of the three different-sized RNAs into three separate virions (**Fig. 1A**). The particles are very unstable in plant sap, can be easily deformed, and the virus concentration in leaf tissue is low. This renders ilarviruses difficult to purify in good quality and sufficient quantity, and has led to the sigla of the genus which was derived from *i*sometric *la*bile *r*ingspot viruses

Ilarviruses possess a tripartite, positive-sense single-stranded RNA genome encapsulated by a coat protein (CP) of approx 25–30 kDa ***(3)***. A schematic drawing of the genome organization and position of the open reading frames is

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

**Table 1**
**Species in the Genus *Ilarvirus***

| Species (acronym) | Subgroup | Taxonomic status |
|---|---|---|
| American plum line pattern (APLPV) | 5 | Approved |
| Apple mosaic (ApMV) | 3 | Approved |
| Asparagus virus 2 (AV-2) | 2 | Approved |
| Citrus leaf rugose (CiLRV) | 2 | Approved |
| Citrus variegation (CVV) | 2 | Approved |
| Elm mottel (EMoV) | 2 | Approved |
| Humulus japonicus (HJV) | 9 | Approved |
| Hydrangea mosaic (HdMV) | 8 | Approved |
| Lilac ring mottle (LRMV) | 7 | Approved |
| Parietaria mottle (PMoV) | 10 | Approved |
| Prune dwarf (PDV) | 4 | Approved |
| Prunus necrotic ringspot (PNRSV) | 3 | Approved |
| Spinach latent (SpLV) | 6 | Approved |
| Tobacco streak (TSV) | 1 | Type member |
| Tulare apple mosaic (TAMV) | 2 | Approved |

The list contains the species according to **ref. *1***

shown in **Fig. 1B**. The two larger genomic RNAs (1 and 2) are monocistronic and encode nonstructural proteins involved in viral replication ***(5)***. In contrast, RNA-3 is bicistronic, encoding a polypeptide presumably required for cell-to-cell movement at the 5' proximal end and the viral CP at the 3' distal end ***(5–7)***. The putative movement protein is translated directly from RNA-3; the CP is expressed from a subgenomic RNA-4, which is colinear with the 3' end of RNA-3 and also becomes encapsidated Ilarviruses are not as intensively studied at the molecular level as other viruses with tripartite genomes, such as alfalfa mosaic virus (AlMV), and brome mosaic virus (BMV); nevertheless, some sequence data became available recently (**Table 2**). So far, however, no complete ilarvirus genome sequence has been published. Some information of ilarvirus molecular biology has been deduced from results obtained for the single member of the next-closest related genus, *Alfamovirus,* alfalfa mosaic virus (AMV).

A unique property of ilarviruses, as well as AMV, which separates them from the other genera of the bromoviridae, is the necessity of CP or CP subgenomic mRNA to initiate infection ***(12)***. This phenomenon, referred to as genome activation, has been studied in most detail with AMV ***(13)***. The genome activation depends on the interaction of the N-terminus of the CP with the 3'-untranslated region of the viral genomic RNAs ***(13)*** Moreover, the respective

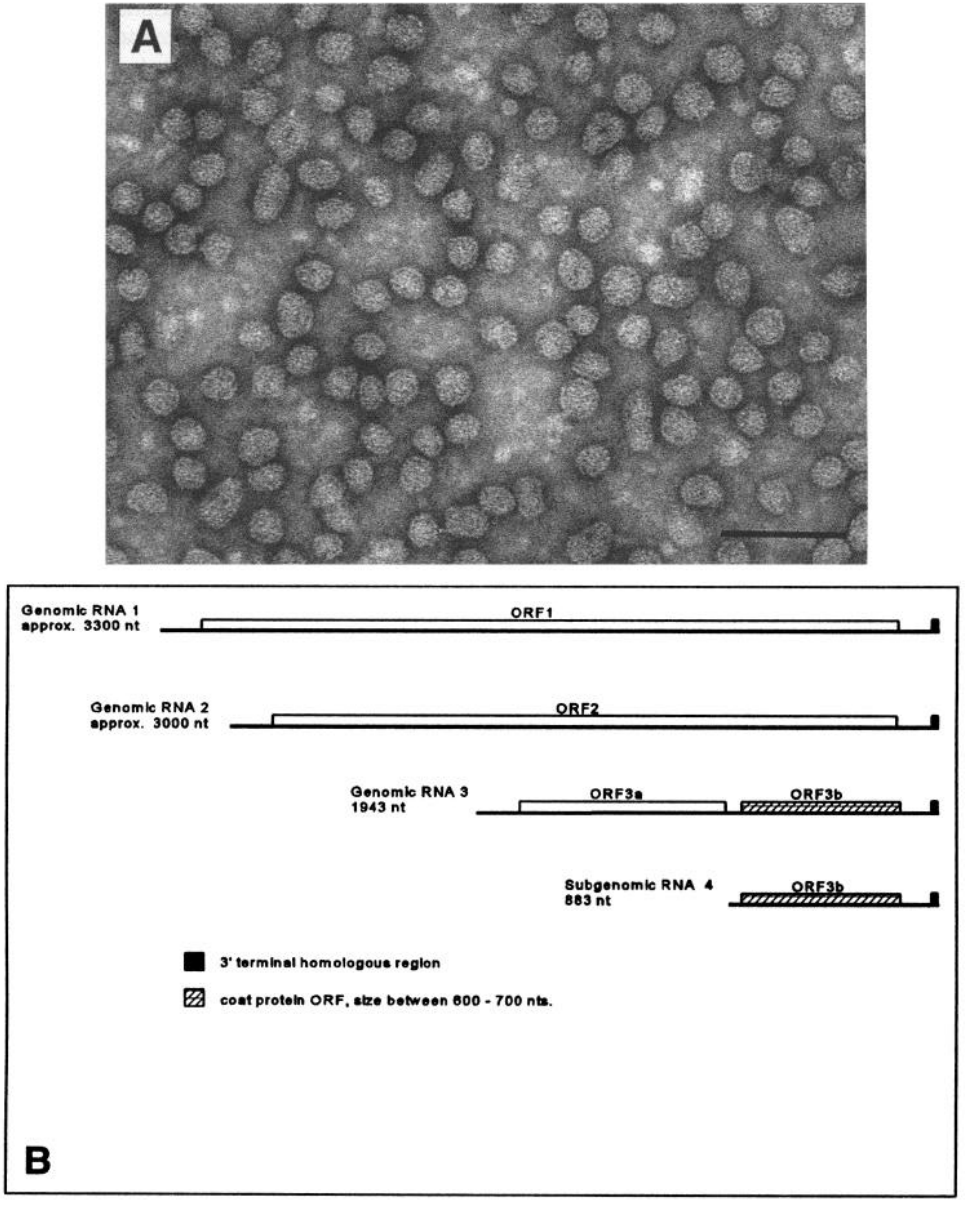



Fig. 1. Morphology and genome organization of ilarviruses. **(A)** Electron micrograph of purified ApMV. Differently sized virions are clearly recognized. Bar = 100 nm. **(B)** Schematic drawing of the genome organization of ilarviruses.

**Table 2**
**Sequence Information Available for Ilarviruses**

| Virus | RNA segment | Accession no for sequence[a] |
|---|---|---|
| Prunus necrotic ringspot | RNA-3 | L 38823 Hammond *(6)* |
| Apple mosaic | RNA-3, RNA-4 | U15608 (Shier et al , unpublished), L03726 *(8)*, U03857 *(9)*[b] |
| Tobacco streak | RNA-3 | X00435 *(10)* |
| Prune dwarf | RNA-4 | L28145 *(11)* |
| Citrus variegation | RNA-3 | U17389 *(7)* |
| Citrus leafrugose | RNA-2; RNA-3 | U17726 *(5)*; U17390 *(7)* |
| Lilac ring mottle | RNA-3 | U17391 *(20)* |

[a]The corresponding references are given in parentheses
[b]This sequence was published as ApMV sequence, but probably is really PNRSV according to sequence comparisons (*see* **refs. *6*** and ***8***)

CPs of several ilarviruses and AMV are freely exchangeable in the process of genome activation. For example, the TSV genome can be activated by AMV CP and vice versa, although they have no apparent sequence similarity *(14)*. Obviously, this shared function depends only on the secondary and tertiary structure of the CP and the viral genomic RNAs, which allow the recognition and interaction between the protein and the viral RNA, even in heterologous combinations *(15)*.

## 2. Materials

### 2.1. Equipment (see Note 1)

1 Centrifuge for Eppendorf (Netheler-Hinz Gmbh, Hamburg, FRG) tubes
2 Low-speed centrifuge with swingout rotor
3 High-speed centrifuge, like Sorval (Newtown, CT)
4 Ultracentrifuge with fixed-angle and swingout rotor
5 ISCO (Columbus, OH) density gradient fractionator
6 pH meter
7. Waring blender
8 Spectrophotometer
9 Pipeting devices like Eppendorf pipets

### 2.2. Buffers and Reagents

1 Inoculation buffer: 0 03*M* HEPES buffer, pH 7 5, or standard virus buffer (*see* **item 2**) without mercaptoethanol, but with diethyldithiocarbamate (DIECA) and 2% polyvinylpyrrolidon (PVP), average mol wt 10,000

2. Buffers for virus purification. 0.03*M* sodium phosphate, pH 8 0, is used as buffer for purified virus and for the homogenization step For the homogenization step, add 0.02*M* 2-mercaptoethanol (2-ME) and 0 02*M* sodium DIECA just before use. **Caution:** 2-ME is harmful, handle it in a fume hood
3. Chemicals for virus purification. Antifoam A solution (Sigma Chemie GmbH, Deisenhofen, FRG)
4. Triton X-100 solution
5. 5*N* NaOH
6. 40% (w/v) Sucrose solution in virus buffer, used as stock solution for preparation of sucrose gradients Gradients are best prepared from four different sucrose concentrations. 10, 20, 30, and 40% In order to prepare linear gradients, divide the nominal volume of the centrifuge tube, minus 1 mL, by four This gives the necessary volume for each concentration per each gradient Pipet the calculated amount of the 10% solution into the tube and underlay the remaining concentrations one after the other with a syringe and long cannula The linear gradient is formed by diffusion during storage of the tubes overnight at 4°C
7. TE buffer 10 m*M* Tris-HCl, 1 m*M* ethylene diaminetetraacetic acid (EDTA), pH 7.5
8. Twofold proteinase K buffer 0 2*M* Tris-HCl, 0 3*M* NaCl, 25 m*M* EDTA, 2% (w/v) sodium dodecyl sulfate (SDS), pH 7.5.
9. TE phenol Phenol saturated with TE buffer, pH of 7 5 **Caution:** Phenol and chloroform are dangerous for your health and the environment, as well as very corrosive Handle them with gloves, protect your eyes with goggles, and work in a fume hood Dispose of residues according to regulations Remove spills, especially in centrifuges, and rotors immediately
10. Phenol·chloroform mix 50% (v/v) TE phenol, 48% (v/v) chloroform, and 2% isoamyl alcohol
11. Chloroform mix chloroform isoamyl alcohol = 24·1 (v/v)
12. 3*M* Sodium acetate Adjusted to pH 5 2 with acetic acid
13. Proteinase K stock solution 10 mg/mL in water Store at –20°C

## 3. Methods (*see* Note 1)

### 3.1. Propagation of Viruses

For most ilarviruses, cucumber *(Cucumis sativus)* is a good source of virus for propagation and purification. Most suitable are the varieties "Riesenschal" and "Lemon"; however, if these varieties are not available, other varieties may be used. These should be tested before use for their sensitivity and the virus titer that is achieved upon inoculation The best condition for cultivating cucumber plants is 24–25°C with an 18-h photoperiod. In uncontrolled conditions, the virus concentration in plants varies seasonally. The most suitable times are spring, autumn, and winter, but additional light is essential. The time required to reach a maximum concentration in cucumber cotyledons is normally 3–6 d after inoculation, but it may take up to 10 d under unfavorable

conditions. For TSV, *Nicotiana tabacum* and *Datura stramonium* are more suitable propagating hosts, but the same procedure as described below can be used for purification

### 3.2. Extraction and Crude Purification

All purification steps should be performed in the cold with precooled buffers, equipment, and rotors. Temperatures between 0 and 4°C are sufficient.

1 Harvest the infected cucumber (*Cucumis sativus*) cotyledons and leaves 3–6 d after inoculation Cool the plant tissues, buffers, and Waring blender to 4°C for 30 min
2 Homogenize the tissue with a Waring blender in extraction buffer (volume equivalent to twice the weight of tissue) for 5 min at maximum speed To prevent excessive foaming when blending, add five drops of antifoam A per 100 g leaf tissue, but this can be omitted if antifoam A is not available
3 Centrifuge the homogenate for 20 min at 1520*g* in a Sorvall GS-3 rotor to remove cell debris. Carefully decant the supernatant into a beaker, and discard pellets
4 Adjust the pH of the supernatant to 4.9 with glacial acetic acid under the control of a pH meter, and incubate at 4°C for 30 min This step will precipitate most chloroplast material, host proteins, and cell debris
5. Centrifuge for 20 min at 11,000*g*, in the same rotor as above, to remove precipitated material Carefully rescue the clear yellow supernatant and adjust the pH of it immediately back to 7 0 with 5*N* NaOH Discard pellets
6 Add Triton X-100 slowly to the supernatant to a final concentration of 2% (v/v) and stir slowly at 4°C for 1 h This step dissolves any remaining membranes and membrane vesicles, and thus prevents their sedimentation during the following ultracentrifugation

### 3.3. First Concentration

Sediment the virus by centrifugation at 105,000*g* for 4 h in a Beckman (Fullerton, CA) Ti-45 rotor and let the pellets resuspend overnight at 4°C in a volume of 0.03*M* sodium phosphate, pH 8.0, equivalent to one-tenth of the original tissue weight. The pellets should be clear and glassy, with a small center of tan-colored, insoluble material. If the pellets are very difficult to resuspend, a Dounce glass homogenizer with a loose fitting piston can be applied to speed up the resuspension process

### 3.4. Fine Purification

1. Centrifuge the resuspended pellet at 16,300*g* for 15 min in a Sorvall HB4 rotor and save the supernatant This will remove most of the opaque portion of the original pellet
2 Sediment the virus again by centrifugation for 3 h at 93,000*g* in a Beckman Ti-70 rotor The resulting pellet should be nearly colorless and the opaque material reduced to a small dot in the center of the pellet. Most of this will be removed by the subsequent low-speed centrifugation

3. Resuspend the pellet by gentle shaking in 200 µL of 0.03*M* sodium phosphate, pH 8.0, per 100 g leaf tissue, and then centrifuge for 10 min at 12,000*g* in a cooled Eppendorf centrifuge. Using a Pasteur pipet, carefully transfer the virus-containing supernatant to a new 1.5-mL centrifuge tube. The preparation at this step, although containing still a little host-plant contamination, is good for viral RNA isolation and subsequent cDNA cloning. The quality can be controlled in an electron microscope and spectrophotometrically. The $OD_{260/280}$ of the preparation should range from 1.45 to 1.60. The concentration can be estimated from the extinction coefficient $E_{260\ nm} \approx 5.1–5.3$, which is equivalent to 1 mg/mL at 1 cm length of the light path.

### 3.5. Sucrose Gradient Centrifugation

If the preparation is to be used for antiserum production, a further purification step by sucrose density gradient centrifugation is recommended. Load at maximum 1 mL, with up to 3 mg of the virus suspension per each preformed sucrose gradient, ranging from 10 to 40% sucrose in 0.03*M* sodium phosphate, pH 8.0, and centrifuge for 4 h at 113,000*g* in a Beckman SW-28 rotor. Carefully collect the virus-containing zones from the gradients with an ISCO density gradient fractionator, according to the absorption profile, monitored at 254 nm. Dilute the virus-containing fractions four times in 0.03*M* sodium phosphate, pH 8.0, and sediment the virus by centrifugation at 123,000*g* for 2.5 h in a Beckman Ti-60 rotor. Resuspend the final pellet in 0.2 mL of the same buffer.

### 3.6. Storage of Purified Virus

For short periods, i.e., a few days, purified virus is stored at 4°C at the highest concentrations possible. For long-term storage, the purified virus should be aliquoted in useful amounts and stored frozen at –80°C or in liquid nitrogen.

### 3.7. Extraction of Viral RNA (see Note 11)

1. Mix the virus preparation with 1 vol of twofold proteinase K buffer and add proteinase K to an end concentration of 400 µg/mL. Incubate the mixture at 37°C for 30 min to digest the viral coat protein.
2. Add 1 vol of TE phenol to the digestion mixture and strongly vortex for 30 s, break the emulsion by a centrifugation for 4 min at room temperature and 8000*g* in a table centrifuge equipped with a swingout rotor. Using a pipet, carefully remove the upper aqueous phase, which contains the RNA.
3. Extract the aqueous phase again, first with phenol:chloroform:mix, and then with the chloroform mix, as described above. Each time, carefully remove the aqueous phase.
4. Add 0.1 vol 3*M* sodium acetate, pH 5.2, and 2.5 vol of ice-cold 100% ethanol to aqueous phase and incubate for at least 30 min at –70°C. This will precipitate the RNA from the solution.

5 Sediment the RNA by centrifugation for 20 min at 4°C and 12,000*g* in a cooled Eppendorf centrifuge
6 Wash the resulting pellet once with 70% ethanol and centrifuge again for 5 min at 12,000*g* as above
7 Dry the pellets for 5 min in a vacuum concentrator and resuspend it in 50 µL sterile water or TE buffer The RNA preparation can be stored under 70% ethanol, as ethanol precipitate at –70°C, for many years The quality and quantity of the RNA should be controlled spectrophotometrically for contaminating proteins (a ratio of absorbance 260/280 nm of ≥2 0 is indicative for the absence of protein) and by agarose gel electrophoresis, where the presence or absence of degradation can be observed

## 4. Notes

1. The equipment list also contains branch names of some manufacturers and equipment. The authors do not want to state that the success of the experiments and methods relies on the use of these specific instruments Instead, it is, of course, possible to use equivalent equipment and inventory The procedures described here were developed for Prunus necrotic ringspot virus, but are also suitable for other ilarviruses (for suggestions for modifications, refer to subsequent notes)
2 The success in purification depends to a large degree on the greenhouse work, i e , the choice and culture of the propagation plants Only well-kept plants develop high titers of virus, and this makes purification easier Second, it is highly advisable to plan the purification schedule carefully Speed is a highly underestimated factor Do not start with more plant material than you can process The bottleneck is usually the first ultracentrifugation step, in which the volume is limited to about 450 mL when using one Ti-45 rotor
3 Choice of propagation plants and their inoculation The varieties of cucumbers or other herbaceous host plants may vary in their suitability for propagation, in case you start anew with work on ilarviruses, make sure you have a suitable variety available The plants should be inoculated at an early developmental stage For cucumbers, this is after the two cotyledones have developed and the first real leaf is barely visible When inoculating with material from woody hosts, add PVP to your inoculation buffer
4 Choice of extraction buffer In some cases, the use of HEPES buffer for extraction and resuspending of the virus seems better than phosphate buffer We have had this experience, especially with PDV, when 0 03*M* HEPES buffer, pH 7 5, worked best In this case, acidify the clarified homogenate with 0 5*M* citric acid and neutralize back later with 0 5*M* NaOH
5 Homogenization step: The homogenization should not last longer than 5 min, otherwise, it will disrupt virus particles Addition of antioxidants 2-ME and DIECA stabilizes the virus particles during the first steps, but are obsolete later
6 First purification steps The yellowish supernatant should be decanted carefully without any green material, which lies between the supernatant and the unsoluble pellet. The green material interferes with the following steps

7 pH adjustments: When adjusting the pH, slowly drop the acidic or basic solution into the extract and stir with a magnetic stirrer to avoid extremely low or high pH and drastic pH changes

8 Other clarification procedures: The method described to clarify the extract is acidification, because the virus can withstand the acidity for the time required to remove the precipitate by centrifugation. If the purification fails, check especially this step, preferably by electron microscopy, to make sure the virus is not lost here. Precipitation of plant material starts around pH 5.2, so maybe a slightly higher pH, as in **Subheading 3.2.**, can help Otherwise the clarification with hydrated calcium phosphate (HCP) has been used to efficiently adsorb host material at the buffer concentration of 0.03*M* ***(16–18)***. The HCP is prepared by adding slightly less than an equal volume of 0 1*M* $CaCl_2$ to a 0 1*M* solution of $Na_2HPO_4$. The white precipitate (HCP) should be washed in distilled water 15–20 times by repeated decantation and resuspension of the precipitate, to remove soluble salts A volume of HCP equivalent to 0 9 of the original weight of tissue is normally needed to clarify the extracts. The buffer concentration of 0.03*M* should be obeyed, since the virus is also adsorbed at lower ionic strength and the host material is not efficiently adsorbed in higher concentrations Finally, ilarviruses can also be concentrated by 10% polyethylene glycol (PEG) 8000 and 1% NaCl (Wang, personal communication) In addition, this would allow the processing of more tissue and larger volumes when the virus titers are low and/or only small-volume ultracentrifuge rotors are available

9 Alternative method to remove host contaminants: Host material may also be efficiently removed from virus preparations by precipitation with an antiserum prepared against host protein The procedure is described in detail by Gold ***(19)***

10. Choice of final purification steps. Usually the sucrose gradient purification is sufficient, however, equilibrium density gradient centrifugation has also been applied, using either CsCl (only after fixation of the purified virus with aldehyde) or $Cs_2SO_4$

11 **Caution:** The general precautions for work with single-stranded RNA, such as sterile buffers, glassware, and use of examination gloves to prevent contamination with RNases, should be strictly obeyed Work at low temperature or on ice is recommended, until otherwise stated

12. When extracting viral RNA with phenol and chloroform, three phases normally appear after centrifugation The upper aqueous phase, containing RNA, should be very carefully removed The intermediate and lower phases, composed of denatured proteins and organic solvent, should be strictly avoided Any trace of them may interfere with the suitability of the RNA preparation for cloning work

13 During the wash steps, it is important to note that the RNA pellet should not be strongly shaken and thereby released from the tube wall, otherwise, the RNA may be easily lost when decanting the solution Gently overturning the centrifuge tube several times may be sufficient.

14 Choice of RNA extraction methods The described method is normally successful and yields RNA with little degradation and is well-suited for cloning steps In

case the RNA looks undegraded according to their electrophonetic pattern, but cloning fails despite a control RNA yielding good results under the same protocol, the following procedure might help Precipitate RNA with ethanol, pellet the RNA, and, after drying in a vacuum concentrator, redissolve the RNA in sterile cacodylate buffer (0 02*M* cacodylic acid, 0 002*M* sodium-EDTA, pH adjusted to 7 2 with 1*N* NaOH) at a concentration of 1 mg/mL Add, per mL of RNA, 0 2 mL cacodylate buffer, 80 µL mercaptoethanol, and 0 2 mL of a sterile 20% SDS solution in water Mix well and incubate for 1–2 min at 60°C in a water bath Thereafter, add 4 mL of TE-phenol and incubate, with occasional shaking, for 10 min in an ice bath Break the emulsion by centrifugation in a low-speed centrifuge with swingout rotor at 8000*g* for 15 min at 20°C, remove the aqueous phase, and precipitate the RNA as described in **Subheading 3.7.** Two wash steps with 70% ethanol should be performed to remove phenol residues **Caution:** Be careful with cacodylate buffer, it is toxic

## References


1. Murphy, F A , Fanquet, C M., Bishop, D. H L , Ghabrial, S A , Jarvis, A W , Martelli, G P., Mayo, M. A , and Summers, M D., eds (1995) Virus Taxonomy Classification and Nomenclature of Viruses Sixth Report of the International Committee on Taxonomy of Viruses *Arch Virol* Suppl 10
2. Fulton, R W (1983) Ilarvirus Group *CMI/AAB Descriptions of Plant Viruses* 275
3. Brunt, A , Crabtree, K , and Gibbs, A , eds (1990) *Viruses of Tropical Plants* CAB International, Wallingford, UK
4. Van Dun, C M P , Overduin, B , van Vloten-Doting, L , and Bol, J F (1988) Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection *Virology* **164,** 383–389
5. Ge, X. and Scott, S W (1994) The nucleotide sequence of citrus leaf rugose ilarvirus RNA-2 *J Gen Virol* **75,** 2841–2846
6. Guo, D , Maiss, E., Adam, G , and Casper, R. (1995) Prunus necrotic ringspot ilarvirus nucleotide sequence of RNA3 and the relationship to other ilarviruses based on coat protein comparison *J Gen Virol* **76,** 1073–1079
7. Scott, S. W and Ge, X (1995) The complete nucleotide sequence of RNA 3 of citrus leaf rugose an citrus variegation ilarviruses *J Gen Virol* **76,** 957–963
8. Alrefai, R H , Shiel, P. J., Domier, L L , D'Arcy, C J , Berger, P H , and Korban, S S (1994) The nucleotide sequence of apple mosaic virus coat protein gene has no homology with other *Bromoviridae* coat protein genes *J Gen Virol* **75,** 2847–2850
9. Sánchez-Navarro, J A and Pallás, V (1994) Nucleotide sequence of apple mosaic ilarvirus RNA 4. *J Gen Virol* **75,** 1441–1445
10. Cornelissen, B J C , Janssen, H , Zuidema, D , and Bol, J F (1984) Complete nucleotide sequence of tobacco streak virus RNA3. *Nucleic Acids Res* **12,** 2427–2437
11. Bachman, E J., Sott, S. W , Xin, G., and Vance, V B (1994) The complete nucleotide sequence of prune dwarf ilarvirus RNA3. implication for coat protein activation of genome replication in ilarviruses. *Virology* **201,** 127–131

12 Bol, J F., van Vloten-Doting, L , and Jaspars, E. M. J (1971) A functional equivalence of top component a RNA and coat protein in the initiation of infection by alfalfa mosaic virus *Virology* **46,** 73–85.
13 Jaspars, E M J (1985) Interaction of alfalfa mosaic virus nucleic acid and protein, in *Plant Molecular Biology,* vol 1 (Davies, J W , ed ), CRC, Boca Raton, FL, pp 155–221
14 Van Vloten-Doting, L (1975) Coat protein is required for infectivity oftobacco streak virus biological equivalence of the coat proteins of tobacco streak and alfalfa mosaic viruses. *Virology* **65,** 215–225.
15 Gonsalves, D and Garnsey, S M. (1975) Infectivity of heterologous RNA-protein mixtures from alfalfa mosaic, citrus leaf rugose, citrus variegation, and tobacco streak viruses *Virology* **67,** 319–326
16 Fulton, R W (1959) Purification of sourcherry necrotic ringspot and prune dwarf viruses *Virology* **9,** 522–535
17 Fulton, R. W (1967) Purification and some properties oftobacco streak and Tulare apple mosaic viruses *Virology* **32,** 153–162
18 Fulton, R W. (1967) Purification and serology of rose mosaic virus *Phytopathology* **57,** 1197–1201
19 Gold, A H (1961) Antihost serum improves plant virus purification *Phytopathology* **51,** 561–565.
20. Scott, S W and Ge, X (1995) The complete nucleotide sequence of RNA 3 of lilac ring mottle ilarviruses J *Gen Virol* **76,** 1801–1806

# 17

# Bromovirus Isolation and RNA Extraction

**Jozef Julian Bujarski**

## 1. Introduction

Bromoviruses are a group of plant single-stranded RNA viruses that belong to the genus *Bromoviridae* Their polyhedral particles of ca 26 nm in diameter have the icosahedral T-3 surface lattice symmetry *(1)*, with 180 identical polypeptides used to build their virion particles Because of the tripartite nature of their genomic RNA, three different particles exist. one containing one molecule of RNA-1, one containing one molecule of RNA-2, and one containing one molecule each of RNA-3 and RNA-4 (*see* **Fig. 1**).

The physicochemical properties of all bromoviruses are similar Their virions are in a stable, compact form at pH between 3 and 6, but swell when the pH is raised above 6.5 *(2)*. They also swell reversibly in presence of $Ca^{2+}$ or $Mg^{2+}$, with concomitant changes in capsid conformation *(3)* Besides coat protein (CP), no lipids or carbohydrates are reported to be contained within bromovirus particles.

Three type members. brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV), and broad bean mottle virus (BBMV), as well as melandrium yellow fleck virus and cassia yellow blotch virus, appear to have the characteristics of bromoviruses *(4)* The bromoviruses have restricted host range: BMV infects mostly *Graminae*, whereas BBMV and CCMV infect a few species in the *Leguminosae*. All three bromoviruses can infect *Nicotiana benthamiana*, a useful virus purification host. In all these hosts, bromoviruses cause systemic mottle or mosaic symptoms, in which they reach and maintain levels of 0.3–3 mg/g of leaf tissue. BMV, BBMV, and CCMV give local lesions in some *Chenopodium* species

The nucleotide sequences of the genomic RNAs of BMV, BBMV, and CCMV are known *(5)*. The BMV replicase proteins 1a and 2a are encoded by

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

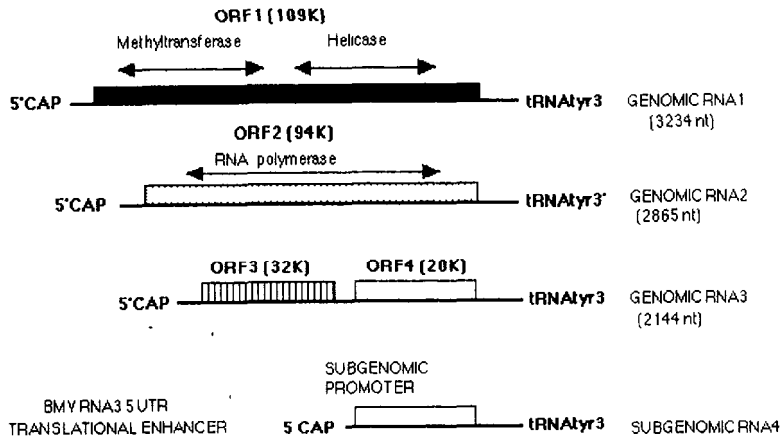



Fig 1 Molecular organization of the brome mosaic virus genome The ORFs are boxed and labeled The domains within ORFs 1 and 2 are marked by double-sided horizontal arrows The noncoding sequences are marked as solid lines The 5' CAP and 3' tRNA-like structures are marked, respectively, at the 5' and 3' RNA ends The size of BMV CP is 27 kDa

RNA-1 and RNA-2, respectively. Protein 1a has at least two domains: one for a putative helicase, and one for putative capping enzyme (guanylyl- and/or methyltransferases), 2a represents the catalytic unit (**Fig. 1**). BMV RNA-3 component encodes the nonstructural movement (3a) protein and the CP. Minus-strand synthesis promoters are located within the 3' noncoding tRNA-like structure region *(6)*. Other sequences responsible for BMV RNA3 replication are within the intercistronic region and at the 5' end The 5' noncoding region also contains internal regulatory motifs. The intercistronic region has the subgenomic RNA-4 promoter *(7)*, as well as signals involved in asymmetric RNA synthesis *(8)*

## 2. Materials

1 Virus inoculation buffer 0.01*M* $NaH_2PO_4$, 0 01*M* $MgCl_2$, pH 6 0 (with NaOH)
2 Virus extraction buffer: 0 5*M* sodium acetate, 0.3*M* acetic acid, 0 01*M* $MgCl_2$, 0 1*M* ascorbic acid (only for BBMV).
3. Virus storage buffer 0.05*M* sodium acetate, 0 01*M* acetic acid, 1 m*M* $Na_2EDTA$, 1 m*M* $MgCl_2$
4. 10X RNA extraction buffer· 0 5*M* glycine, 0 5*M* sodium chloride, 0 1*M* EDTA, pH 9.0
5 RNA loading solution 0 5% bromophenol blue, 0 5% xylene-cyanol, 15% Ficoll dissolved in DEPC-treated water

6 RNA electrophoresis separating buffer (0 5X TBE buffer). 0.045*M* Tris, 0 045*M* boric acid, 0 001*M* EDTA, pH 8.0
7 Polyethylene glycol (PEG) solution· 30% (w/v) of PEG, average mol wt 8000, in water.

## 3. Methods

### 3.1. Virus Propagation

1. Arrange a set of pots filled with a soil mixture (PRO-MIX R6) Equal the top by pressing with another pot
2. Water the soil
3 Put 10–20 barley seeds on top and cover with a 1-cm layer of soil Press with another pot and water
4 Put pots into greenhouse and keep in a full-light condition, below 25°C. If necessary, use artificial light with a photoperiod of 16 h light/8 h dark
5 Prepare virus inoculum by grinding 1 g of infected leaves (taken from another plant) with a mortar and pestle in 2 mL of inoculation buffer Add a 600-mesh Carborundum powder to the inoculum
6 Dust the two- to three-leaf stage barley plants with Carborundum and inoculate the virus by rubbing each leaf with gloved fingers moistened with inoculum The leaves must be rubbed hard, but not hard enough to damage the leaves
7 Using a household sprayer, spray the inoculated plants with water immediately after inoculation to prevent leaf dehydration
8. Keep the inoculated plants in greenhouse for another 2 wk

### 3.2. Virus Purification (see Note 1)

1 Collect those leaves that show systemic symptoms (a mosaic) of BMV infection. Do not collect inoculated leaves
2 Grind infected leaf tissue in chilled mortar using pestle and crushed glass, with 1 mL extraction buffer per gram of tissue (*see* **Note 1**)
3. Transfer into a centrifuge tube (e.g., Corex glass tube or plastic Oakridge tube) and add 0.2 mL chloroform/g tissue, then emulsify by vortexing for 30 s
4. Centrifuge for 5 min at 5000*g* in a low speed centrifuge at 4°C Use a precooled rotor.
5 Remove supernatant, avoiding the interface, and filter it through a coarse filter paper or three layers of Miracloth
6. Add one-third vol of 30% PEG, stir for 1 min, and leave on ice for 30 min
7 Collect the precipitate by centrifugation for 10 min at 12,000*g*
8 Dissolve the pellet in 0 2 mL storage buffer per gram of the original tissue and emulsify again with 0 4 mL of chloroform per 1 mL of virus solution
9 Centrifuge 5 min at 12,000*g* and discard the supernatant
10 Leave the virus pellet on ice overnight in 0.2 mL of storage buffer per gram of the original tissue to resuspend the virus
11. Ultracentrifuge the dissolved pellet for 2 h at 100,000*g*.

12 Remove the supernatant
13. Dissolve pellet (*see* **Note 2**) in the storage buffer and centrifuge for 10 min at 30,000*g*
14. Withdraw the supernatant (discard the pellet) and repeat **steps 11–14**
15. Determine virus concentration by measuring UV light absorption at 260 nm (*see* **Note 3**)
16 Store the virus frozen at –70°C (*see* **Note 4**)

### 3.3. Extraction of Virion RNA (see Note 5).

1. Pipet out 200 µL purified virion preparation into an Eppendorf tube, add 25 µL 10% SDS (w/v), 25 µL 10X RNA extraction buffer, and 250 µL phenol
2 Vortex for 20 s.
3 Centrifuge for 4 min at 14,000 rpm in an Eppendorf microcentrifuge
4 Transfer the upper (aqueous) phase to a new tube, then add 125 µL phenol (*see* **Note 6**) and 125 µL chloroform (*see* **Note 7**).
5 Vortex for 20 s
6 Centrifuge for 4 min at 14,000 rpm
7 Transfer the upper phase to a new tube, then add 250 µL chloroform
8 Vortex for 20 s
9 Centrifuge for 3 min at 14,000 rpm
10. Transfer the upper phase to a new tube and add 2 5 vol ethanol
11 Keep on ice for 30 min
12 Centrifuge for 12 min at 14,000 rpm
13 Discard supernatant, wash the pellet by vortexing 30 s with 200 µL of 70% ethanol
14 Centrifuge for 3 min at 14,000 rpm
15 Discard the supernatant
16. Dry the pellet in SpeedVac (under vacuum) for 3–5 min
17 Dissolve in 50 µL DEPC-treated water.
18 Store the RNA preparation at –70°C (*see* **Note 8**)

### 3.4. Electrophoresis of Virion RNA

1 Melt the stored RNA preparation slowly on ice.
2 Vortex for 10 s
3 Spin down all the droplets by centrifugation for 10 s at 14,000 rpm (e.g., in Eppendorf microcentrifuge)
4. While melting RNA samples, cast a 1% agarose gel in an autoclaved 0.5X TBE buffer The electrophoresis tank should be pretreated with hot 1% SDS for 15 s, followed by immediate rinsing three times with sterile distilled water
5 Load 0 1–0 5 µg RNA to each well in a sterile RNA loading solution
6. Run electrophoresis for 1–3 h, depending on the length of the unit (the longer the unit, the better separation of the RNA segments) at 100 V
7. Stain the gel with ethidium bromide (0 001% [w/v] solution) for one-half hour, then examine the gel under UV lamp.

## 4. Notes

1 This protocol works well for BMV and CCMV In the case of BBMV, especially if the virus is extracted from broad bean leaves, the ascorbic acid should be added to virus extraction buffer The basic protocol can be scaled-down for virus extraction from mg quantities of leaf tissue In this case, the centrifugation steps are accomplished using Eppendorf tubes and a microcentrifuge, and ultracentrifugation steps are omitted The entire procedure should be done on ice or in cold room (*see also* **ref. *9***)

2 Pellet should appear clear and glassy

3. 1 $OD_{260}$ corresponds to 0.2 mg of the virus. The purity of virion RNA samples can be estimated by measuring the ratio of UV absorbency at 260/280 A ratio of 1 8–2 0 indicates that the RNA sample contains no, or only residual, amounts of proteins

4 The virus will preserve its biological activity for years when stored in storage buffer at –70°C To avoid thawing and refreezing of the samples, store the virus in small aliquots Virus yield 0 5–5 mg/g infected leaf tissue

5. The procedure gives good yields of viral RNA for three bromoviruses The RNA purification procedure should be done entirely on ice or in a cold room

6 High-quality phenol saturated with 0 1*M* Tris buffer, pH 7 0 (e g , from Gibco-BRL) should be used for RNA isolation High-quality water (double-distilled) should be treated with DEPC overnight at 37°C, followed by autoclaving

7 High-quality chloroform stabilized with isoamyl alcohol (mix 1 vol of isoamyl alcohol with 24 vol of chloroform) is recommended for RNA isolation

8 Store the RNA preparations in small aliquots of DEPC-treated water at –20 to –80°C The RNA preparation is stable for years Avoid thawing and refreezing of the samples Yield· 0 03–0 1 mg/g infected leaf tissue

## References

1 Speir, J A , Munshi, S , Wang, G , Baker, T. S., and Johnson, J E (1995) Structures of the native and swollen forms of cowpea chlorotic mottle virus determined by X-ray crystallography and cryo-electron microscopy *Structure* **3,** 63–78

2 Bancroft, J B , Hills, G J , and Markham, R. (1967) A study of self-assembly process in a small spherical virus formation of organized structures from protein subunits in vitro *Virology* **31,** 354–379

3 Jacrot, B (1975) Studies on the assembly of a spherical virus II The mechanism of protein aggregation and virus swelling. *J Mol Biol* **95,** 433–446

4 Lane, L. (1979) Bromovirus Group. No 215 in CMI/ABB Descriptions of Plant Viruses, Commonw. Mycol Inst Assoc. Appl Biologists, Kew, Surrey, UK

5 Bujarski, J J , Nagy, P. D , and Flasinski, S. (1994) Molecular studies of genetic RNA-RNA recombination in brome mosaic virus, in *Advances in Virus Research*, vol 43 (Maramorosch, K , Murphy, F , and Shatkin, A., eds )

6 Ahlquist, P , Bujarski J J , Kaesberg, P , and Hall, T C (1984) Localization of the replicase recognition site within brome mosaic virus RNA by hybrid-arrested RNA synthesis *Plant Mol Biol* **3,** 37–44

7 Miller, W A , Dreher, T W , and Hall, T. C. (1985) Synthesis of brome mosaic virus subgenomic RNA in vitro by internal initiation on (–) sense genomic RNA *Nature* **313,** 68–70
8 Marsh, L E , Pogue, G. P , Huntley, C C , and Hall, T. C (1991) Insight to replication strategies and evolution of (+) strand RNA viruses provided by brome mosaic virus. *Oxford Surv. Plant Mol Cell Biol* **7,** 297–334
9. Lane, L (1986) Propagation and purification of RNA plant viruses. *Methods Enzymol* **118,** 687–696

# 18

# Cucumovirus Isolation and RNA Extraction

**Marilyn J. Roossinck and P. Scott White**

## 1. Introduction

The *Cucumoviruses* are tripartite (+) sense RNA viruses in the *Bromoviridae* family of plant viruses The genus includes cucumber mosaic virus (CMV, the type species), peanut stunt virus (PSV), and tomato aspermy virus (TAV) (reviewed in **refs.** *1* and *2*) A fourth member, bean distortion mosaic virus (BDiMV), has also been proposed as a new species *(3)*. CMV is further divided into two subgroups, based on hybridization data and serology *(2)*. PSV probably also contains at least two subgroups, and perhaps as many as six *(4)* CMV has the broadest host range of any known plant virus, infecting up to 1000 species of plants, and inducing a very wide range of symptoms in infected plants *(2)*. PSV has a narrower host range, infecting predominantly solanaceous plants and legumes, and TAV is predominantly restricted to solanaceous plants and composites *(1)* Symptoms for all of the cucumoviruses may include stunting, mosaic, and leaf distortion, and may range from mild to severe.

The *Cucumoviruses* encode at least four proteins (outlined in **Fig. 1**), and possibly a fifth *(5)*. RNA-1 and RNA-2 contain large open reading frames (ORFs) encoding the 1a and 2a proteins, respectively, the viral components of the replicase complex *(6,7)*. A second ORF is found on RNA-2 of all reported cucumoviruses, but the gene product for this ORF has not been shown for all strains of CMV *(5)*. RNA-3 encodes the 3a protein, necessary for virus movement, and recently shown to interact with plant plasmadesmata *(8)* The 1a, *(9)*, the 2a *(10)*, and the coat protein (CP) *(11)* have also been implicated in virus movement. The CP is translated from a subgenomic RNA-4, which is collinear with the 3' portion of RNA-3 The genomic RNAs are packaged individually, with the subgenomic RNA-4 packaged along with RNA 3 *(12)*. Some strains of CMV, especially Subgroup II strains, contain additional smaller RNA species

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

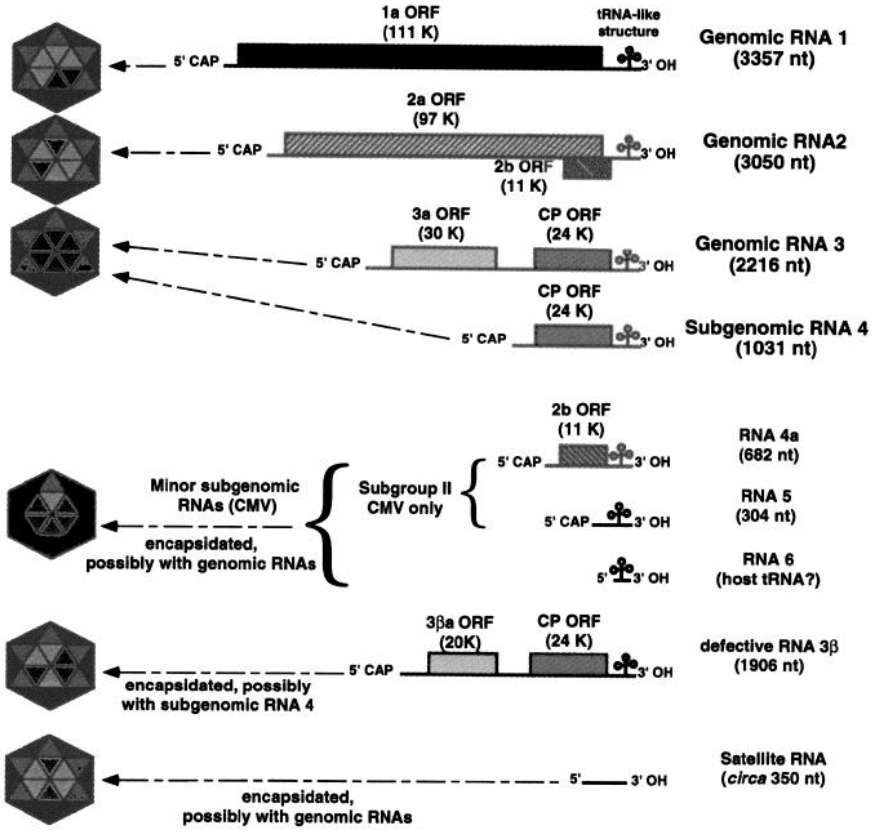



Fig. 1. *Cucumovirus* genome organization. CMV-Fny, a subgroup I CMV, genomic RNAs 1, 2, and 3, and subgenomic RNA 4 are shown. Genomic segments of other *Cucumovirus* species are of similar sizes. The minor subgenomic segments 4a and 5 are found in some subgroup II CMV isolates, and RNA 6 is found in isolates from both CMV subgroups and PSV. Other subgenomic RNAs have been observed in several PSV isolates. RNAs 1, 2, and 3 are sufficient for establishing systemic infection. A defective RNA (3β) has been found associated with CMV-Fny, and satellite RNAs have been observed with isolates of CMV and PSV.

***(2)***. In addition, defective RNAs have been found associated with one strain of CMV, which are also packaged ***(13)***, and satellite RNAs are often associated with CMV and with PSV (reviewed in **ref.** ***14***).

The virions of CMV, TAV, and most strains of PSV are approx 30 nm in diameter, with icosahedral symmetry and a T number of 3 ***(15)***. The robinia strain of PSV is reported to have a larger virion, of approx 40 nm ***(16)***, although the CP sequence is highly similar to other PSV strains (F. García-Arenal and J.

J Bernal, personal communication, and our unpublished observation). Virion integrity is dependent upon protein–RNA interactions, and the virions are partially permeable, making them subject to degradation on exposure to low concentrations of ribonucleases *(17)*. In addition, the virions are not stable to freezing. Hence, all steps of virus purification are done at 4°C The half-life of purified virions is about 2 wk to 1 mo. Long-term storage of cucumoviruses is most reliable in the form of viral RNA, which is highly infectious, and very stable at temperatures of –20°C. Alternatively, many strains may be stably stored as virions in buffer C, plus 50% glycerol, at –20°C

The method for virus purification described here is based on that published by Lot et al *(18)*, with modifications for PSV as in **ref. *19***, for TAV as in *(20)*, and for the universal buffer system as in *(3)* *Nicotiana tabacum* (tobacco), *N clevelandii*, or *N benthamiana* are suitable hosts for propagation of most cucumoviruses, although some strains of PSV do not infect tobacco CMV may also be propagated in *Cucurbita pepo* (zucchini squash), or *Cucumis sativus* (cucumber), and PSV may be propagated in *Vigna unguiculata* (cowpea) The RNA extraction method is based on that of Palukaitis and Zaitlin *(21)* RNA is very susceptible to degradation by ribonucleases. **Caution:** All glassware used for RNA should be baked overnight at 160°C, and gloves should be worn to prevent contamination by ribonucleases found on the skin

Purification of cucumoviruses involves thoroughly grinding infected tissue in a buffer appropriate to the particular virus, followed by a chloroform extraction, and high-speed centrifugation through a sucrose cushion Virus pellets are then resuspended and contaminants are removed by a low-speed centrifugation, followed by a second high-speed centrifugation. For very pure virus, a sucrose gradient may be utilized, but for most purposes the sucrose cushion is sufficient The buffers given in **Subheading 3.1.** are standard buffers for CMV purification. Other buffers used for other cucumoviruses, as well as a universal buffer system, are given in **Subheading 4**. Viral RNA is readily purified from virus by the addition of SDS, and three extractions with phenol:chloroform

## 2. Materials

### 2.1. Virus Purification

All buffers should be used at 4°C. Buffers are given for CMV. (*See* **Notes 1–4** for buffers for other cucumoviruses )

1 CMV buffer A 0 5*M* sodium citrate, pH 7 0, 5 m*M* EDTA, 0 5% thioglycolic acid (stored in liquid form at –20°C, and added just before use).
2 CMV cushion I· 0 5*M* sodium citrate, pH 7.0, 5 m*M* EDTA, 10% sucrose
3. CMV buffer B 5 m*M* sodium borate, pH 9.0, 5 m*M* EDTA, 2% Triton X-100
4 CMV cushion II· 5 m*M* sodium borate, pH 9 0, 5 m*M* EDTA, 10% sucrose.
5 CMV buffer C 5 m*M* sodium borate, pH 9 0, 5 m*M* EDTA Autoclave

### 2.2. RNA Extraction

1. VEBA *(21a)* 0.2*M* Tris, pH 8.5, 1*M* NaCl, 1% SDS, 2 m*M* EDTA. Autoclave and store at room temperature. VEBA must be warmed slightly before use, to resuspend the SDS.
2. Phenol:chloroform, 5:1, saturated with water (store at 4°C). Phenol should be ultrapure quality, or should be redistilled. **Caution:** Use care in handling, phenol is extremely caustic.
3. NAE: 0.3*M* sodium acetate, pH 6.0, 0.1 m*M* EDTA. Autoclave and store at room temperature.
4. 0.1 m*M* EDTA, pH 8.0, autoclave and store at room temperature.

## 3. Methods

### 3.1. Virus Purification

All centrifugation values are given as maximum relative centrifugal force (RCF). *See* **Note 5** for handling of glassware.

1. Harvest fresh plant leaf tissue, removing major ribs and stems. Use as soon as possible, or store for a short period at 4°C. Avoid any freezing of infected tissue.
2. Weigh tissue and place in a chilled blender jar (*see* **Note 6**). For each gram of tissue, add 1 mL of buffer A and 1 mL of cold (4°C) chloroform. Blend until thoroughly homogenized, about 2 min.
3. Transfer homogenate to a centrifuge bottle, and centrifuge at 15,000*g* at 4°C for 10 min.
4. Filter the aqueous phase through dampened Miracloth (*see* **Notes 7** and **8**). Transfer the filtrate to ca. 25-mL ultracentrifuge tubes, and underlay with 5 mL of cushion I. It is most convenient to use thick-walled polycarbonate tubes, because the volume can be varied, and they are available with sealable screw caps. The ultracentrifugation should be completed as soon as possible. Do not keep the virus in buffer A for more than a few hours.
5. Centrifuge at 212,000*g* for 1.5 h (*see* **Note 9**) at 4°C.
6. Pour off the supernatant, and add 4–5 mL of buffer B to each pellet. Allow the pellets to sit at 4°C overnight.
7. Vortex the pellets in buffer B briefly, pool the samples, and stir at 4°C for 2 h.
8. Centrifuge the stirred samples at 7500*g* at 4°C for 10 min. Pour off the supernatant immediately to an ultracentrifuge tube. Underlay with 5 mL of cushion II.
9. Centrifuge as in **step 5** (*see* **Note 10**). Pour off supernatant and add 2–5 mL of buffer C (depending on size) to the pellets. Allow the pellets to sit at 4°C overnight.
10. Virus can be stored in buffer C, or used for RNA extraction. To quantitate the virus yield, measure the $OD_{260}$. CMV has an extinction coefficient of 5 *(22)*. Virus yields vary from 100 to 1 g/kg of tissue, dependent on the strain of virus. The virus may be stored at –20°C in 50% glycerol.

### 3.2. RNA Extraction

1 Vortex the virus pellets in buffer C, and add an equal volume of VEBA Immediately pour the mixture into a tube containing an equal volume of phenol·chloroform For total extraction volumes of 20 mL or less, a 50-mL disposable tube may be used with a wrist-action shaker For larger volumes, use a beaker or flask
2 Shake or stir the extraction for 15 min at room temperature
3 Centrifuge the emulsion at top speed in a tabletop centrifuge for 10 min
4 Remove the aqueous phase to a fresh tube containing the same volume of phenol chloroform Also, remove most of the fluffy interface Repeat **steps 2** and **3**
5 Remove only the aqueous phase to a fresh tube containing phenol chloroform, and repeat **steps 2** and **3** above
6 Remove only the aqueous phase to a clean 30 mL Corex tube, and add 2–2 5 vol of absolute ethanol Store at –20°C for several hours or overnight
7. Centrifuge the ethanol precipitate at 12,000*g* at 4°C for 30 min
8 Pour off the supernatant and dry the pellets under vacuum
9. Resuspend the pellets in 5 mL of NAE per tube, with vortexing If the RNA pellet is very large, freezing and thawing can help resuspend the pellet Concentrations of RNA can be as high as 10 mg/mL before saturation in water.
10. Add 12 5 mL absolute ethanol and store at –20°C as in **step 6** Repeat **steps 7** and **8**
11 Resuspend the pellets in 0 5 mL of NAE, and transfer to an Eppendorf tube Add 1 mL of absolute ethanol for a final precipitation
12 Centrifuge at top speed in a microcentrifuge for 10 min Pour off supernatant, dry pellets under vacuum, and resuspend in 0 5 mL of 0 1 m*M* EDTA Dilute 10 µL into 1 mL of water, and measure the OD at 260 and 280 nm The 260/280 ratio should be 2 The extinction coefficient for RNA is 25 ***(23)*** The virus is about 18% RNA Store the RNA at –20°C

## 4. Notes

1 Alternate buffers for PSV are: buffer A, 0 1*M* sodium citrate, pH 7 0, 20 m*M* EDTA, 0 1% thioglycolic acid (v/v, add just before use), extraction is done with 2 mL buffer A and 2 mL chloroform per gram of tissue, initial pellets are resuspended in water, and 0 1 vol of CMV buffer A, plus Triton X-100 to 2% is added before stirring, the second pellet is resuspended in water, and 0 1 vol of CMV buffer A is added for storage; cushion I, 0.1*M* sodium citrate, pH 7 0, 1 m*M* EDTA, 10% sucrose, cushion II, 50 m*M* sodium citrate, pH 7 0, 0 5 m*M* EDTA, 10% sucrose
2 Alternate buffers for TAV are· buffer A, 0 1*M* sodium or potassium phosphate, pH 7 0, 0 5% thioglycolic acid, buffer B, 20 m*M* phosphate, pH 7 0, 1% Triton X-100, buffer C, 20 m*M* phosphate, pH 7.0, or, if viral RNA is to be extracted, virus may be resuspended in water (avoid EDTA in the final resuspension buffer), cushion I, 0.1*M* phosphate, pH 7 0, 10% sucrose, cushion II, 20 m*M* phosphate, pH 7 0, 10% sucrose

3 Alternate universal buffers, which work well for all cucumoviruses tested, are buffer A, 0 1 sodium citrate, pH 7 0, 20 m*M* EDTA, 0 1% sodium diethyldithiocarbamate (DIECA) (w/v, add just before use), 0 1% (v/v) 2-mercaptoethanol, buffer B, 10 m*M* Tris, pH 7 0, 0 5 m*M* EDTA, 2% Triton X-100, buffer C, 10 m*M* Tris, pH 7 0, 0 5 m*M* EDTA (for TAV use 20 m*M* phosphate, *see* **Note 2**), cushion I, 10 m*M* Tris, pH 7 0, 0 5 m*M* EDTA, 2% Triton X-100, 20% sucrose, cushion II, 10 m*M* Tris, pH 7 0, 0 5 m*M* EDTA, 20% sucrose For alternate universal buffers, use 2 mL buffer A and 2 mL chloroform per gram of tissue for extraction, ultracentrifugations are done at 150,000*g* for 3 5 h

4 One strain of CMV, M-CMV, requires a unique method for purification Because of a modification of the CP, M-CMV is unstable in the presence of chloroform and of EDTA *(24)* M-CMV is extracted as follows buffer A, 0 1*M* dibasic sodium phosphate, 0 1% thioglycolic acid (add just before use), and 0 1% DIECA (add just before use), extract in 3 mL buffer A per gram of tissue and filter the homogenate through two layers of cheesecloth and two layers of Miracloth Centrifuge the filtrate at 15,000*g* at 4°C for 10 min. Add Triton X-100 to 2%, and stir at 4°C for 15 min Centrifuge at 78,000*g* for 2 h Resuspend in 0 1*M* dibasic sodium phosphate, as for CMV resuspension Centrifuge at 5500*g* at 4°C for 10 min, and pour the supernatant into an ultracentrifuge tube Underlay with 5 mL 0 1*M* dibasic sodium phosphate, 10% sucrose, and centrifuge, as above Resuspend final pellet in 10 m*M* sodium borate, pH 8 0

5 To avoid crosscontamination, especially of satellite RNAs, all plastic and glassware must be thoroughly cleaned after each use It is preferable to bake all glass and metal components of blender jars, tubes, flasks, and stir bars overnight at 160°C Items that cannot be baked should be soaked for 2 h in 0 1*M* sodium hydroxide, followed by thorough rinsing

6 For less than 20 g of tissue, a polytron works best for homogenization Alternatively, small amounts of tissue can be ground in buffer A in a mortar and pestle, squeezed through several layers of cheesecloth, and extracted with chloroform by shaking in the centrifuge bottle, before low-speed centrifugation For 20–50 g of tissue, use a small blender jar (500-mL capacity), for 50–250 g of tissue, use a large blender jar (1000-mL capacity)

7 For very large-scale preparations, virus may be concentrated by precipitation with polyethylene glycol (PEG) prior to the first ultracentrifugation step Replace the thioglycolic acid in buffer A with 40 m*M* 2-mercaptoethanol (S Flasinski, personal communication) Add 10 g of PEG 8000 for every 90 mL of extract in buffer A Stir at 4°C for 45 min Centrifuge at 15,000*g* at 4°C for 10 min Drain the pellets thoroughly, because residual PEG may cause the resuspended virus to precipitate Resuspend the pellet in buffer B, using one-fourth to one-third of the original extraction volume Stir for 45 min at 4°C Centrifuge at 7500*g* at 4°C for 10 min The virus will be in the supernatant, and the pellet should be very small, a large pellet indicates that the residual PEG has reprecipitated the virus In this case, save the pellet, and resuspend it again in buffer B Continue the purification with the second ultracentrifugation

8 For small samples, or if the first pellets are very small, volumes can be reduced and the ultracentrifugation done in 10-mL tubes Underlay 10-mL tubes with 2 mL of cushion.
9. The time and speed for ultracentrifugation may vary depending on the type of cushion used (20% sucrose, 10% sucrose, or no cushion) In general, centrifugation should be done at speeds ranging from 150,000*g* for 3 5 h to 212,000*g* for 1.5 h If problems arise with centrifuge tubes cracking, lower the speed and lengthen the time used
10 For very pure virus (e g., for rabbit injection for antibodies), a 5–25% linear sucrose gradient in 20 m*M* sodium phosphate, pH 7 0, may be used Centrifuge in a swinging-bucket rotor at 100,000*g* for 3 h. The viral band can be visualized and collected by its light-scattering property A maximum of ca 5 mg of virus can be loaded onto one 34-mL gradient The pooled virus bands are diluted fourfold in 20 m*M* sodium phosphate, pH 7 0, and pelleted by ultracentrifugation at 212,000*g* at 4°C for 2 h For antisera, the virions must be stabilized with formaldehyde treatment *(25)*

## References


1. Kaper, J M and Waterworth, H E. (1981) Cucumoviruses, in *Handbook of Plant Virus Infections and Comparative Diagnosis* (Kurstak, E , ed ), Biomedical, North Holland, pp 258–332
2 Palukaitis, P, Roossinck, M J , Dietzgen, R G , and Francki, R I B (1992) Cucumber mosaic virus, in *Advances in Virus Research* (Maramorosch, K , Murphy, F A , and Shatkin, A J , eds ), Academic, San Diego, pp 281–348
3 White, P S , Morales, F J , and Roossinck, M J , (1995) Interspecific reassortment in the evolution of a cucumovirus *Virology* **207,** 334–337
4 Richter, J , Haack, I , Wesemann, M , and Beczner, L (1987) Differenzierung den Erdnußstauche-Virus (peanut stunt virus) in 6 Serotypen *Arch Phytopathol Pflanzenschutz, Berlin* **23,** 127–133
5 Ding, S -W , Anderson, B J , Haase, H R , and Symons, R H (1994) New overlapping gene encoded by the cucumber mosaic virus genome *Virology* **198,** 593–601
6 Hayes, R. J. and Buck, K W (1990) Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase. *Cell* **63,** 363–368
7 Nitta, N , Takanami, Y , Kuwata, S , and Kubo, S (1988) Inoculation with RNAs 1 and 2 of cucumber mosaic virus induces viral RNA replicase activity in tobacco mesophyll protoplasts *J Gen Virol* **69,** 2695–2700
8 Ding, B , Li, Q , Nguyen, L , Palukaitis, P, and Lucas, W J (1995) Cucumber mosaic virus 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants *Virology* **207,** 345–353
9 Gal-On, A , Kaplan, I , Roossinck, M J , and Palukaitis, P (1994) The kinetics of infection of zucchini squash by cucumber mosaic virus indicate a function for RNA 1 in virus movement *Virology* **205,** 280–289.
10 Carr, J P, Gal-On, A , Palukaitis, P, and Zaitlin, M (1994) Replicase-mediated resistance to cucumber mosaic virus in transgenic plants involves suppression of

both virus replication in the inoculated leaves and long-distance movement *Virology* **199,** 439–447.

11 Taliansky, M E and García-Arenal, F (1995) Role of cucumovirus capsid protein in long-distance movement within the infected plant *J Virol* **69,** 916–922

12 Lot, H and Kaper, J M (1976) Further studies on the RNA component distribution among the nucleoproteins of cucumber mosaic virus *Virology* **74,** 223–226

13 Graves, M V and Roossinck, M J (1995) Characterization of defective RNAs derived from RNA 3 of the Fny strain of cucumber mosaic cucumovirus *J Virol* **69,** 4746–4751

14 Roossinck, M J , Sleat, D , and Palukaitis, P (1992) Satellite RNAs of plant viruses: structures and biological effects *Microbiol Rev* **56,** 265–279

15 Finch, J T , Klug, A., and VanRegenmortel, M H V (1967) The structure of cucumber mosaic virus. *J Mol Biol* **24,** 303–305.

16 Schmelzer, K (1971) Robinia mosaic virus *CMI/AAB Descriptions of Plant Viruses* **65**

17 Habili, N and Francki, R I B (1974) Comparative studie on tomato aspermy and cucumber mosaic viruses II Virus stability *Virology* **60,** 29–36

18 Lot, H , Marrou, J , Quiot, J B , and Esvan, C (1972) Contribution à l'étude de virus de la mosaique du concombre (CMV) II Méthode de purification rapide du virus *Ann Phytopathol* **4,** 25–38

19 Diaz-Ruiz, J R , Kaper, J M , Waterworth, H E , and Devergne, J C (1979) Isolation and characterization of peanut stunt virus from alfalfa in Spain *Phytopathology* **69,** 504–509

20 Palukaitis, P and Roossinck, M J. (1995) Variation in the hypervariable region of cucumber mosaic virus satellite RNAs is affected by the helper virus and the initial sequence context *Virology* **206,** 765–768

21 Palukaitis, P and Zaitlin, M (1984) Satellite RNAs of cucumber mosaic virus characterization of two new satellites *Virology*, **132,** 426–435

21a Palukaitis, P and Symons, R H (1980) Purification and characterization of the circular and linear forms of chrysanthemum stunt viroid *J Gen Virol* **46,** 477–489

22 Francki, R I B , Randles, J W , Chambers, T C , and Wilson, S B (1966) Some properties of purified cucumber mosaic virus (Q strain) *Virology* **28,** 729–741

23 Sambrook, J , Fritsch, E F , and Maniatis, T (1989) *Molecular Cloning A Laboratory Manual*, 2nd ed Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

24 Mossop, D W , Francki, R I. B., and Grivell, C J (1976) Comparative studies on tomato aspermy and cucumber mosaic viruses. V Purification and properties of a cucumber mosaic virus inducing severe chlorosis *Virology* **74,** 544–546

25 Francki, R I B and Habili, N (1972) Stabilization of capsid structure and enhancement of immunogenicity of cucumber mosaic virus (Q strain) by formaldehyde *Virology* **48,** 309–315

# 19

# Nepovirus Isolation and RNA Extraction

**K. Roger Wood**

## 1. Introduction

Viruses of the nepovirus genus are classified within the family of *Comoviridae*, along with the comoviruses and the fabaviruses. At the time of writing, there are some 27 confirmed members of the genus, with eight or so viruses that, following further investigation, may be confirmed as members *(1)*. These viruses infect a very wide range of plant species, producing symptoms that include mottles, mosaics, ringspots, and systemic necrosis, often infections are symptomless, particularly in the later stages. All members are transmitted through soil by free-living nematodes, principally of *Longidorus* or *Xiphinema* species, feeding on roots. They are also transmitted and disseminated through seed and pollen of both crop species and weeds It is the combination of a mode of transmission (*ne*matode) and virus morphology (*po*lyhedral) that provides the name of the genus

They have a worldwide distribution, although, as a result of their transmission via nematodes, many individual members of the genus have a rather restricted geographical range This may be wider if seed or vegetatively propagated material has been more widely distributed. Diseases occur in many important crop species, including soybean in the United States (tobacco ringspot nepovirus, TobRSV) and raspberries in the UK (raspberry ringspot nepovirus; RRV). Arguably, the most important is the disease of grapevine caused by grapevine fanleaf nepovirus (GVFV).

Nepoviruses have bipartite, single-stranded RNA genomes of messenger sense [ss(+)RNA], each species independently packaged into icosahedral particles of T = 1 symmetry *(2)* approx 28 nm in diameter, each particle contains 60 copies of a single capsid protein, with an approximate $M_r$ of 55 kDa. The larger of the two genomic RNAs, RNA-1 has $M_r$ approx $2.8 \times 10^6$ (**ref. *3***;

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

8100–8400 nucleotides), while the smaller of the two, RNA-2, is much more variable in size between members of the genus, ranging in size from approx 1 4 to 2.4 × $10^6$ (3400–7200 nucleotides). The genus can be divided into two subgroups, according to the size of the RNA-2 component, those in Subgroups I and II having RNA-2 smaller or greater than 5400 nucleotides, respectively.

Virus particles, isolated from infected tissue, are usually, though not always, of three types. top (T), middle (M), and bottom (B), named originally according to their position following separation by sucrose gradient ultracentrifugation Those viruses with RNA-2 molecules in the larger size range have T particles consisting of capsid protein only, M containing RNA-2, and B particles containing RNA-1 Those with RNA-2 components in the smaller size range have particles containing two molecules of RNA-2, in addition to those containing a single copy. In these cases, the sedimentation coefficient of the particles containing two copies overlaps that of particles containing RNA-1, so that the B component particles are of two types ***(4)***. Often, T particles are not present In addition, multiple copies of a satellite RNA, if present, may also be encapsidated by viral coat protein.

Each RNA has a small virus-encoded protein (VPg) attached covalently to the 5'-terminus (the presence of which is essential for infectivity), and a poly(A) sequence at the 3'-terminus Each has a single open reading frame (ORF), encoding a polyprotein that is proteolytically cleaved to produce a series of smaller functional proteins

The genome organization of a representative nepovirus, tomato black ring (TBRV), is indicated in **Fig. 1A** and **C**. Nucleotide sequence data ***(5)***, and comparison with the genome sequences of the related como- and picornaviruses, suggests that RNA-1 ($M_r$ 2.69 × $10^6$) encodes proteins of $M_r$ 92, 72, 60, 23, and 2 3 kDa, which would be expected to be processed from a primary transcript of 254 kDa Proteins identified from either in vitro translation experiments ***(6, 7)***, or from the investigation of proteins produced in vivo ***(8)***, are also indicated in **Fig. 1B** and **D**. The sizes of the proteins in **Fig. 1B** and **D** are those derived from electrophoretic data, which may differ from the values predicted from the nucleotide sequence.

The capsid protein is represented by amino acids 838–1348 at the 3'-terminus of the primary translation product of RNA-2 (**ref. *9***; 1.66 × $10^6$), a polyprotein of 1357 amino acids, $M_r$ 150 kDa. It is produced from the 150-kDa polyprotein by cleavage to a 59-kDa protein, which then loses nine C-terminal amino acids, to become the 57-kDa capsid protein, $M_r$ 55,888 ***(10)***.

The function of some, but not all, the other proteins has been established. The 92-kDa protein is a component of the viral replicase, the 23-kDa protein is a protease, and VPg (2.3 kDa) becomes attached to the genomic RNAs. VPg is cleaved from the 120-kDa protein, although neither this protein nor the 92- and

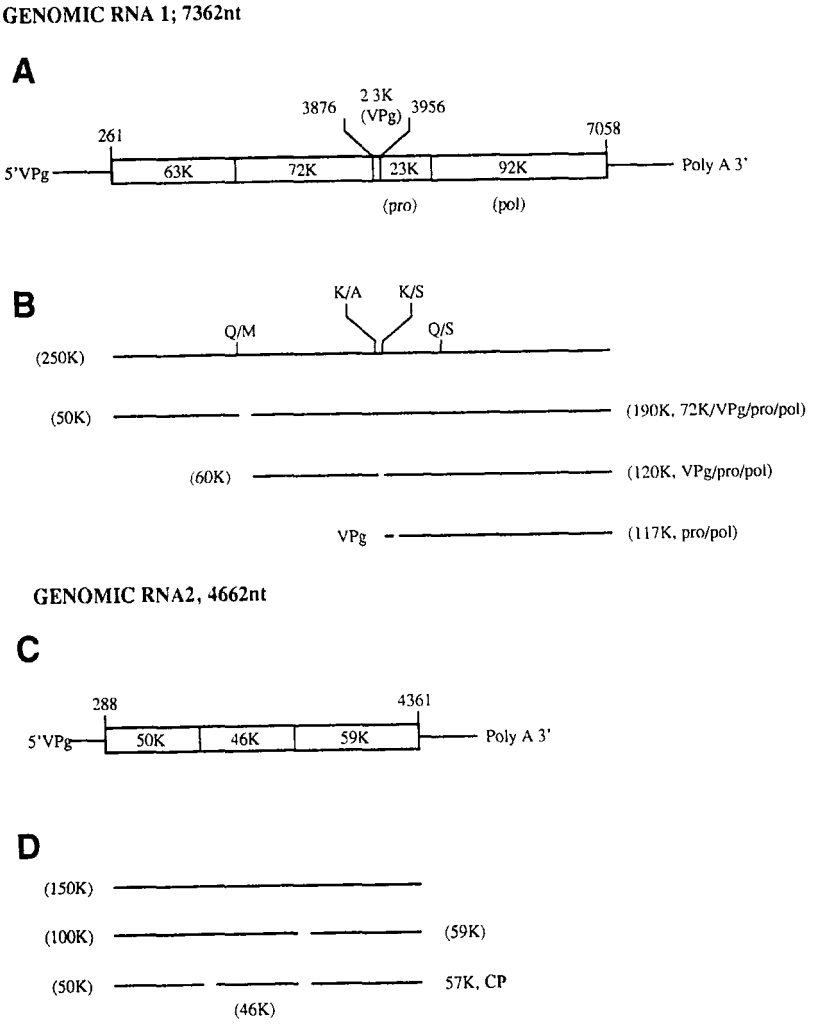



Fig 1 The proposed genome organization of TBRV RNA-1 and RNA-2 The polypeptides indicated in **(A)** and **(C)**, with putative cleavage sites, are those predicted from sequence data The sizes of the polypeptides in **(B)** and **(D)** are deduced from electrophoretic analysis of polypeptides identified in vitro or in vivo (Data used with permission from **refs. *5–10.***)

23-kDa proteins have been detected as separate entities either in vitro or in vivo. Both the 120-kDa and 117-kDa products have proteolytic activity ***(7)***, and in vitro translation experiments suggest that the activity of this protease is necessary for processing of genome-encoded products. In the absence of RNA-1, for example, the 150-kDa RNA-2-encoded polyprotein is not processed.

There is also complete sequence data available for the RNA-2 species of a number of other nepoviruses, including Hungarian grapevine chrome mosaic *(11)*, GFLV *(12)*, RRV *(13)*, and tomato ringspot (TomRV, **ref. *14***) The last of these has an unusually large RNA-2 component (7273 nucleotides), which is partially accounted for by a long 3' noncoding region The capsid protein sequence occupies the same position in the polyprotein sequence as that of other nepoviruses Although the genome of strawberry latent ringspot virus has much in common with the nepoviruses, the viral capsid contains two proteins, $M_r$ 29 and 44 kDa; the position of this virus within the *Comoviridae* family remains to be resolved *(15)*

Nepoviruses are usually stable and relatively easy to purify. The virus purification procedure outlined below utilizes the standard procedures of sap clarification using an organic solvent, virus precipitation with polyethylene glycol (PEG), and several cycles of differential centrifugation. It is essentially that of Mayo et al. *(16)*, and works well for many of the nepoviruses For others, however, the use of chloroform to clarify sap extracts may be preferred (*see* **Subheading 4.**) RNA is obtained from virions using a standard phenol·chloroform procedure

## 2. Materials

### 2.1. Virus Purification

1 Carborundum (F 500, Carborundum, Manchester, UK)
2 Butter muslin
3 Omnimixer (Camlab, Cambridge, UK)
4 Butan-1-ol (*n*-butanol; Merck, Luttenworth, UK)
5 PEG 6000 (Merck)
6 Homogenization buffer 0 07*M* sodium phosphate buffer, pH 7 0, 0 01*M* in di-Na EDTA and containing 0 1% (v/v) mercaptoacetic acid
7 Resuspension buffer A 0 07*M* sodium phosphate buffer, pH 7 0
8 Resuspension buffer B 10 m*M* Tris-HCl, 50 m*M* NaCl, 1 m*M* di-Na EDTA, pH 7 5

### 2.2. RNA Purification

1 Wrist-action shaker (Fisher Scientific, Loughborough, UK)
2. Sodium dodecyl sulfate (SDS, Merck)
3 Phenol mix· phenol, containing 0 1% 8-hydroxyquinoline, saturated with resuspension buffer B
4 Chloroform mix chloroform and isoamyl alcohol mixture (24·1, [v/v]), saturated with resuspension buffer B
5 Sodium acetate
6 70% (v/v) Ethanol

## 3. Method

### 3.1. Virus Purification

1. Dust approx 20 *Nicotiana tabacum* (cv White Burley) plants (at approximately the 4–5 leaf stage), with carborundum, and mechanically inoculate with virus inoculum (*see* **Notes 1** and **2**)
2. Maintain the plants at approx 23°C for 7–10 d (*see* **Note 3**)
3. Collect the inoculated leaves (ca. 200–250 g), and homogenize thoroughly in homogenization buffer (1 2, w/v) using an Omnimixer (*see* **Notes 4–6**)
4. Filter the homogenate through two layers of muslin, make 8.5% (v/v) with *n*-butanol, and stir gently for 30 min (*see* **Note 7**)
5. Centrifuge at 10,000*g* for 20 min and discard the pellet
6. Make the supernatant solution 10% (w/v) in PEG 6000 and 0 17*M* sodium chloride
7. Stir gently for 1 h
8. Centrifuge at 10,000*g* for 20 min Discard the supernatant solution
9. Resuspend the pellet in resuspension buffer A (10% original volume of the homogenate, **step 3**, *see* **Note 8**)
10. Allow the mixture to stand overnight.
11. Centrifuge at 10,000*g* for 10 min and discard the pellet
12. Centrifuge the supernatant solution at 150,000*g* for 2 h, discard the supernatant solution
13. Resuspend the pellet in a small volume (ca 2–3 mL) of resuspension buffer A
14. Repeat **steps 11** and **12**
15. If the virus is to be used for RNA extraction, resuspend the pellet in 2–3 mL resuspension buffer B, alternatively, use buffer A
16. Dilute a small sample 100X in the same buffer used for final resuspension and assess the virus concentration spectrophotometrically, assuming that a suspension of 1 mg/mL has an absorption of 10 (260 nm, 1 cm path length) (*see* **Note 9**).

### 3.2. RNA Purification

1. Make the virus suspension (ca 5 mg/mL in buffer B) 1% (w/v) in SDS, and vortex briefly (*see* **Note 10**).
2. Add an equal volume of phenol mix and shake vigorously for 5 min at room temperature on a wrist-action shaker (*see* **Note 11**)
3. Add chloroform mix (same volume as phenol mix) and shake again for 5 min at room temperature
4. Add ca. 50 µL silicone grease and centrifuge at 2500*g* for 5 min (*see* **Note 12**)
5. Collect the (upper) aqueous phase, and make 0 2*M* in sodium acetate
6. Add 2 vol of ethanol and allow to stand for at least 2 h at –20°C to precipitate the RNA
7. Centrifuge at 12,000*g* for 20 min
8. Add 70% (v/v) ethanol (approximately twice the volume of the original virus suspension) and vortex for a few seconds

9 Centrifuge at 2500*g* for 1 min and remove the ethanol
10 Repeat this ethanol washing at least twice more
11 Either store the RNA pellet in 70% (v/v) ethanol at 4°C or resuspend the RNA in distilled water and store at –80°C until required

## 4. Notes

1 Tobacco is a suitable source of TobRSV-infected tissue for purification purposes However, for this and other nepoviruses, several other hosts are appropriate, *Nicotiana clevelandii* is suitable for many, but for TomRV, for example, cucumber or squash may be more appropriate
2 The yield of virus to be expected will vary, depending on the virus to be used For TobRSV, a conservative estimate would be 50 mg/kg; 5–20 mg/kg is typical A convenient amount of tissue to process is 250–400 g, but this can clearly be adjusted if large amounts of virus or viral RNA are required
3 The time at which tissue is harvested is not absolutely critical, but, in our hands, 7–10 d after inoculation has provided highest yields from inoculated tissue, when plants are maintained under the conditions described
4 Harvested tissue may be processed immediately, or stored at –80°C for several months, until required If stored frozen, the tissue must be brought close to 4°C before homogenization
5 TobRSV is stable for several days at room temperature in sap However, unless otherwise stated, it is preferable to perform all steps at 4°C to maintain maximum infectivity and integrity of the viral RNA
6 An Omnimixer top-drive macerator is ideal for tissue homogenization, all liquid is effectively contained Bottom-drive blenders of the Waring type are also satisfactory
7 Clarification using *n*-butanol is appropriate for many of the nepoviruses A useful alternative, which also gives good results, is to use chloroform (e g , **refs.** ***17*** and ***18***) In this method, tissue is homogenized in homogenization buffer and chloroform (1 1 1 [w/v/v]), and the mixture allowed to stand for 30 min The mixture is then centrifuged at 10,000*g* for 20 min, and the lower layer discarded The supernatant solution is then processed as indicated (**steps 6–16**)
8 A critical aspect of this purification process, that is common to all procedures which include pelleting of the virus by high-speed centrifugation, is efficient resuspension of the virus pellet Since some viruses are susceptible to aggregation, this can be difficult, although nepoviruses do not pose as severe a problem as do some other groups of viruses, (e g , potyviruses) I use glass rods and rubber policemen (crude but effective) An additional modification is to sediment virus particles onto a cushion of Maxidens (Nycomed), a high-density perfluorinated liquid that is immiscible with water
9 The method described here provides a mixture of the two genomic RNAs If it is intended to use RT-PCR to clone the sections of the genome required, then this is all that is required However, if purified preparations of RNA-1 and RNA-2 individually are required, additional steps will need to be included

(*see* Chapter 26) If the two genome components are encapsidated in particles which differ in their sedimentation coefficient, then it is possible to obtain preparations of virus particles enriched in either of the two RNAs by sedimentation through 10–40% sucrose in 0.07*M* phosphate buffer, pH 7 0 Further purification may be necessary, particularly if the RNA-2 component of the virus in question is in the lower size range The M component, containing RNA-2, may be subjected to a second centrifugation through a sucrose gradient; RNA-1-containing B particles may be purified by isopycnic centrifugation in cesium chloride *(19)*. RNA may then be extracted from pooled fractions containing the appropriate component The preparations of RNA-1 and RNA-2 thus obtained may then be further purified by a final electrophoretic separation through agarose or polyacrylamide gel To obtain a sample of RNA-1 without contaminating RNA-2, a preliminary particle separation will almost certainly be necessary

10. A mixture of pronase and SDS has been used by some in RNA isolation (**Subheading 3.2., step 1**) However, if infectious RNA is required, the use of a proteolytic enzyme is not recommended There is a possibility that the 5'-terminal VPg will be removed from the viral RNAs
11 It is possible to use a prepared phenol:chloroform mix in **Subheading 3.2., step 2** However, my experience is that a two-stage procedure as described gives better results
12 The addition of a small quantity of silicone grease (**Subheading 3.2., step 4**), which collects at the interface between the organic and aqueous phases during centrifugation, greatly facilitates the collection of the aqueous layer without contamination

## References

1 Murant, A F (1981) Nepoviruses, in *Handbook of Plant Virus Infections and Comparative Diagnosis* (Kurstak, E , ed ), Elsevier/North Holland, Amsterdam, pp 197–238
2. Roberts, I. M (1988) The structure of particles of tobacco ringspot nepovirus evidence from electron microscopy *J Gen Virol* **69,** 1831–1840
3 Murant, A. F , Taylor, M , Duncan, G. H , and Raschke, J H (1981) Improved estimates of molecular weight of plant virus RNA by agarose gel electrophoresis and electron microscopy after denaturation with glyoxal *J Gen Virol* **53,** 321–332
4 Mayo, M A , Harrison, B D , Murant, A F., and Barker, H (1973) Cross-linking of RNA induced by ultra-violet irradiation of particles of raspberry ringspot virus. *J Gen Virol* **19,** 155–159
5 Greif, C , Hemmer, O , and Fritsch, C (1988) Nucleotide sequence of tomato black ring virus RNA-1. *J Gen Virol* **69,** 1517–1529
6 Demangeat, G , Greif, C , Hemmer, O , and Fritsch, C (1990) Analysis of the in vitro cleavage products of the tomato blackring virus RNA-1-encoded 250K polyprotein *J Gen Virol* **71,** 1649–1654.

7 Hemmer, O , Greif, C , Dufourcq, P , Reinbolt, J , and Fritsch, C (1995) Functional characterisation of the proteolytic activity of the tomato black ring nepovirus RNA-1-encoded polyprotein *Virology* **206,** 362–371
8 Demangeat, G , Hemmer, O , Reinbolt, J , Mayo, M A , and Fritsch, C. (1992) Virus-specific proteins in cells infected with tomato black ring nepovirus evidence for proteolytic processing in vivo *J Gen Virol* **73,** 1609–1614
9 Meyer, M , Hemmer, O , Mayo, M A , and Fritsch, C. (1986) The nucleotide sequence of tomato black ring virus RNA-2 *J Gen Virol* **67,** 1257–1271
10 Demangeat, G , Hemmer, O , Fritsch, C , Le Gall, O , and Candresse, T (1991) In vitro processing of the RNA-2-encoded polyprotein of two nepoviruses tomato black ring virus and grapevine chrome mosaic virus *J Gen Virol* **72,** 247–252
11 Brault, V , Hibrand, L , Candresse, T , Le Gall, O , and Dunez, J (1989) Nucleotide sequence and genetic organisation of Hungarian grapevine chrome mosaic nepovirus RNA2 *Nucleic Acids Res* **17,** 7809–7819
12 Serghini, M A , Fuchs, M , Pinck, M , Reinbolt, J , Walter, B , and Pinck, L (1990) RNA2 of grapevine fanleaf virus sequence analysis and coat protein cistron location *J Virol* **71,** 1433–1441
13 Blok, V C , Wardell, J , Jolly, C A , Manoukian, A , Robinson, D J , Edwards, M L , and Mayo, M A (1992) The nucleotide sequence of RNA-2 of raspberry ringspot nepovirus *J Gen Virol* **73,** 2189–2194
14 Rott, M E , Tremaine, J H , and Rochon, D M (1991) Nucleotide sequence of tomato ringspot virus RNA-2 *J Gen Virol* **72,** 1505–1514
15 Kreiah, S , Strunk, G , and Cooper, J I (1994) Sequence analysis and location of capsid proteins within RNA 2 of strawberry latent ringspot virus *J Gen Virol* **75,** 2527–2532
16 Mayo, M. A , Barker, H , and Harrison, B D. (1982) Specificity and properties of the genome linked proteins of the nepoviruses *J Gen Virol* **59,** 149–162
17 Salazar, L F and Harrison, B D (1978) The relationship of potato black ringspot virus to tobacco ringspot and allied viruses *Ann Appl Biol* **90,** 387–394
18 Rezaian, M A and Francki, R I B (1973) Replication of tobacco ringspot virus I Detection of a low molecular weight double stranded RNA from infected plants *Virology* **56,** 238–249
19 Harrison, B D and Barker, H. (1978) Protease-sensitive structure needed for infectivity of nepovirus RNA *J Gen Virol* **40,** 711–715

# 20

# Comovirus Isolation and RNA Extraction

**Joan Wellink**

## 1. Introduction

Comoviruses are small, icosahedral viruses with a diameter of approx 28 nm (**Fig. 1**) Presently the genus *Comovirus* is comprised of 15 different viruses, of which the type species, cowpea mosaic virus (CPMV), is the most thoroughly studied *(1,2)* All viruses are transmitted by beetles and have a rather narrow host range. Most comoviruses have legumes as their natural hosts and usually cause mosaic or mottling symptoms Comoviruses are mechanically transmissible, and can replicate to high levels in infected cells. Purified preparations of comoviruses consist of two, sometimes three, distinguishable particles, which can be separated by centrifugation on sucrose density gradients. These particles are designated as bottom (B), middle (M), and top (T) component, corresponding to their position in the centrifuge tube The B- and M-component are nucleoprotein particles, each containing a segment of the single-stranded, bipartite RNA genome (denoted RNA-1 and RNA-2, respectively); T-component consists of empty protein shells Both B- and M-components, or their RNAs, are necessary for infectivity *(3)*

The protein capsids of B-, M-, and T-component are identical, consisting of 60 copies each of a large (L) and small (S) coat protein, the observed differences in sedimentation coefficient and density are exclusively caused by differences in RNA content. Top components do not seem to have a specific function in virus infectivity and may be regarded as a side product of the viral assembly process. The amount of T-component produced varies greatly among different comoviruses and even among different isolates of the same virus, and seems to be dependent on the condition for growth

Molecular weights of RNA-1 and RNA-2 reported for different comoviruses are all in the range of 2.0–2 4 x $10^6$ and 1.2–1.45 × $10^6$, respectively. The

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

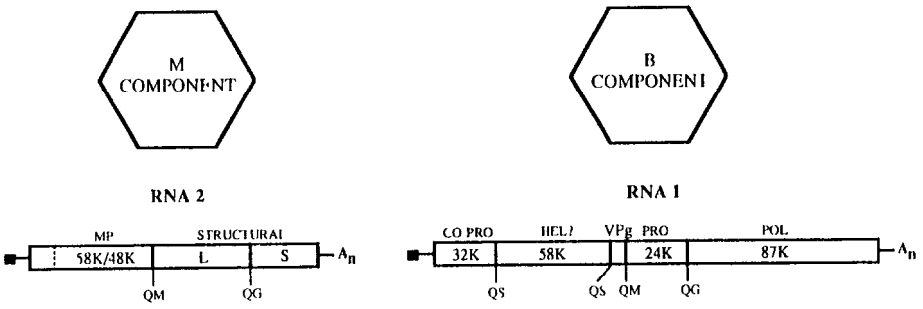



Fig 1 Genetic organization of the CPMV genome The icosahedral virus particles consist of two components, denoted B and M, that contain RNA-1 and RNA-2, respectively The ORFs in the RNA molecules are indicated with open bars and VPg with a black square The RNAs are translated into polyproteins that are proteolytically processed at six specific sites, indicated below the ORFs, into several stable intermediate and nine final cleavage products These latter products are indicated in the ORFs Functions of the different domains in the polyproteins are shown above the ORFs with the following abbreviations MP, movement protein, CO-PRO, cofactor required for proteinase, HEL?, putative helicase, PRO, proteinase, POL, RNA-dependent RNA polymerase

genomic RNAs of the comoviruses have a small protein covalently linked to their 5' end (denoted VPg *V*iral *P*rotein genome-bound) and a poly(A) tract at their 3' end (**Fig. 1**).

All comoviruses sequenced so far contain a single, long open reading frame (ORF) that occupies over 80% of the length of the RNA. Expression of both RNAs involves the production of large polyproteins, from which several smaller proteins are derived by proteolytic processing through the action of a viral proteinase (**Fig. 1**) The RNA-2 of all comoviruses tested are translated in vitro into two carboxy coterminal polyproteins, because of initiation of translation at a second in-frame AUG codon. For CPMV, this has also been shown to occur in infected cells A total of 15 intermediate and final cleavage products have been identified in cells infected with CPMV as a result of processing at six specific dipeptide sequences (**Fig. 1**). CPMV RNA-1 is able to replicate independently from RNA-2 in cowpea protoplasts, and all proteins coded for by RNA-1 are needed for viral RNA replication. RNA-2, on the other hand, is needed for the infection of whole plants and specifies the two capsid proteins and the movement protein (**Fig. 1**)

The purification and extraction methods described below have been developed for CPMV. With minor modifications, these methods should also be applicable for other comoviruses (*see* **Subheading 4.**). The precipitation of

viruses with polyethylene glycol was first described by Hebert ***(4)***, the RNA extraction protocol is based on the procedure as described by Zimmern ***(5)***.

## 2. Materials

1 Stock 0 1*M* phosphate solution, pH 7 0
2 Sucrose cushion 40% sucrose in 0 1*M* phosphate buffer, pH 7 0.
3 Extraction buffer 0 02*M* Tris-HCl, pH 7 6, 0 2*M* NaCl, 2m*M* EDTA, 4% SDS
4 Phenol Equilibrated to a pH >8 0 and containing 0 1% hydroxyquinoline
5 Chloroform used for the RNA extraction chloroform isoamyl alcohol (24 1)

## 3. Methods

### 3.1. Purification of Virus

1 Homogenize infected leaves with 0 1*M* phosphate buffer, pH 7 0 (0°C, 2mL/g leaf tissue) in a blender or a mortar, and press the homogenate through two layers of Miracloth (*see* **Notes 1–3**)
2 Clarify the extract by centrifugation at 15,000*g* for 20 min
3 Stir the supernatant for 1 min with 0 7 vol of chloroform and *n*-butanol (1 1) and centrifuge at 1000*g* for 5 min (*see* **Note 4**)
4 Remove the clear aqueous layer and add to it polyethylene glycol 6000 (PEG) to a final concentration of 4% (w/v) and NaCl to a concentration of 0 2*M* Stir the mixture at room temperature to dissolve the PEG and NaCl, and incubate for 1 h (*see* **Note 5**)
5 Collect the precipitate by centrifugation at 20,000*g* for 15 min and resuspend the pellet in 0.01*M* phosphate buffer, pH 7 0 (0 5 mL/g leaf tissue) (*see* **Note 6**)
6 Clarify the suspension by centrifugation at 10,000*g* for 15 min at 4°C (*see* **Note 4**)
7 Layer the suspension on a sucrose cushion and sediment the virus by centrifugation at 150,000*g* for 3 h at 4°C (*see* **Note 4**)
8 Resuspend the virus pellet in sterile distilled $H_2O$ (pH >5 2) and clarify the suspension by centrifugation at 10,000*g* for 15 min at 4°C If the infectivity has to be preserved add phosphate buffer, pH 7.0, to a final concentration of 10 m*M* (*see* **Note 7**)
9 To determine the virus concentration, measure the OD at 260 nm A 1 mg/mL CPMV suspension has an OD of 8 1 (*see* **Note 8**)

### 3.2. Extraction of Viral RNA

1 Dilute the virus suspension to a concentration of 10 mg/mL or less and add 1 vol of extraction buffer and 2 vol of phenol Vortex for 3 min (*see* **Note 9**)
2. Add 2 vol of chloroform, vortex for 2 min and separate the phases by centrifugation at 10,000*g* for 2 min (*see* **Note 10**)
3 Repeat the extraction of the aqueous layer two times with 1 vol phenol and 1 vol chloroform added simultaneously
4 Precipitate the RNA with 0 1 vol of 3*M* NaAc, pH 5 2, and 2 2 vol ethanol, and wash the pellet with 70% ethanol

## 4. Notes

1 Other methods, as described by Van Kammen *(6)*, and Bruening and Agrawal *(7)*, result in lower yields of virus.
2 A pH of 6.0 has been used to prevent formation of brown-colored substances *(8)*
3 It may be important to carry out the initial steps in the purification in rapid succession to decrease the tendency of the virus to aggregate *(8)*
4 During virus purification, **steps 3, 6,** and **7** of **Subheading 3.1.,** can be omitted This shortens the procedure; however, the virus suspension will contain more contaminants
5 Higher amounts of PEG have been used (up to 10%) This may be useful when the virus concentration is low.
6 To prevent aggregation and formation of brown-colored substances, it may be helpful to use a phosphate buffer containing 2 m*M* EDTA and 2 m*M* β-mercaptoethanol in this step
7 When the virus pellet is not clear, the virus suspension can be further purified by an extra high-speed centrifugation in 0 1*M* phosphate buffer (150,000*g* for 2 h) and repetition of **Subheading 3.1., step 8**
8 Prior to RNA extraction, virus components can be separated on sucrose density gradients *(9)* and/or by isopycnic centrifugation on, for example, CsCl or Nycodenz (**refs.** *10* and *11*, *see also* Chapter 26). A 1-mg/mL suspension of purified CPMV B- and M-component has an $OD_{260}$ of 10 0 and 6.2, respectively
9. Other methods to extract the RNA from comovirus particles include the disruption of the particles in a detergent solution by heating *(12)*, and treatment by proteinase K *(13)* After RNA extraction, the RNAs can be separated and further purified by sucrose density gradients, as described, for example, by Van Klootwijk et al (**ref.** *12*, *see also* Chapter 26)
10. Re-extraction of the phenol·chloroform layer with 0 5X extraction buffer containing 0 1% SDS can be used to improve the yield of viral RNA

## References

1 Eggen, E and Van Kammen, A (1988) RNA replication in comoviruses, in *RNA Genetics*, vol 1 (Ahlquist, A Holland, J , and Domingo, E , eds ), CRC, Boca Raton, FL, pp. 49–69
2 Goldbach, R and Wellink, J. (1996) Comoviruses molecular biology and replication, in *The Plant Viruses*, vol 5 (Harrison, B D and Murant, A F , eds ), Plenum, New York, pp 35–76.
3 Van Kammen, A (1968) The relationship between the components of cowpea mosaic virus. I. Two ribonucleoprotein particles necessary for the infectivity of CPMV *Virology* **34,** 312–318
4 Hebert, T. T (1963) Precipitation of plant viruses by polyethylene glycol *Phytopathology* **53,** 362–369
5 Zimmern, D (1975) The 5' end group of tobacco mosaic virus RNA is $m^7 G^{5'}ppp^{5'}Gp$ *Nucleic Acids Res* **2,** 1189–1201
6 Van Kammen, A (1967) Purification and properties of the components of cowpea mosaic virus *Virology* **31,** 633–642

7 Bruening, G. and Agrawal, H O (1967) Infectivity of a mixture of cowpea mosaic virus ribonucleoprotein components *Virology* **32,** 306–320
8 Oxelfelt, P. (1976) Biological and physicochemical characteristics of three strains of red clover mottle virus *Virology* **74,** 73–80
9 Goldbach, R Rezelman, G , and Van Kammen, A (1980) Independent replication and expression of B-component RNA of cowpea mosaic virus *Nature* **286,** 297–300
10 Bruening, G (1977) Plant comoviruses two component systems, in *Comprehensive Virology*, vol. 11 (Fraenkel-Conrad, H. and Wagner, R R , eds ), Plenum, New York, pp. 55–141
11. Holness, C L , Lomonossoff, G P , Evans, D , and Maule, A J (1989) Identification of the initiation codons for translation of cowpea mosaic virus middle component RNA using site-directed mutagenesis of an infectious cDNA clone *Virology* **172,** 311–320
12 Van Kootwijk, J , Klein, I , Zabel, P , and Van Kammen, A (1977) Cowpea mosaic virus RNAs have neither $m^7$GpppN. . nor mono-, di- or triphosphates ar their 5' ends. *Cell* **11,** 73–82
13 Hiebert, E and Purcifull, D E. (1981) Mapping of the two coat protein genes on the middle RNA component of squash mosaic virus (comovirus group) *Virology* **113,** 630–636

# 21

# Carmovirus Isolation and RNA Extraction

**Juana Díez, Jose F. Marcos, and Vicente Pallás**

## 1. Introduction

The Carmovirus group is named after *car*nation *mo*ttle virus (CarMV), its type member. Carmoviruses have icosahedral particles about 30 nm in diameter that sediment at 120–130 S. They are also characterized by a monopartite positive-sense, single-stranded RNA genome of $M_r$ 1.4–1.6 × $10^6$ and a single capsid protein of $M_r$ 36,000–46,000 *(1)*

There is relatively little information on epidemiology and control of the majority of carmoviruses, though some of them, including CarMV, melon necrotic spot (MNSV), or cowpea mottle (CPMoV) viruses, have significant economic importance. A common characteristic of carmoviruses is their relative facility of transmission by mechanical inoculation. Many carmoviruses have been reported to be transmitted to plants through soil, either in the absence of vectors (CarMV or galinsoga mosaic virus, GMV) or by soil-inhabiting fungi (the case of cucumber soil-borne virus, CSBV, and MNSV). For only one member, pelargonium flower break virus (PFEV), transmission facilitated by thrips has been demonstrated.

CarMV is, together with turnip crinkle virus (TCV), the best-studied of carmoviruses. The complete sequence of CarMV consists of 4003 nucleotide residues *(2)*. CarMV RNA contains a 69-nucleotide 5' leader sequence before the first AUG and a 288-nucleotide 3' untranslated region. Sequence analysis of the CarMV genome revealed the presence of five open reading frames (**Fig. 1**) that could potentially encode proteins of 27 (p27), 86 (p86), 98 (p98), 7 (p7), and 38 (p38) kDa. p86 and p98 would be synthesized from the first AUG by readthrough at two different UAG amber termination codons, respectively. p86 contains the GDD motif characteristic of RNA-dependent RNA polymerases of positive-strand RNA viruses. The sequence coding for the CarMV

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G. D. Foster and S. C. Taylor © Humana Press Inc., Totowa, NJ

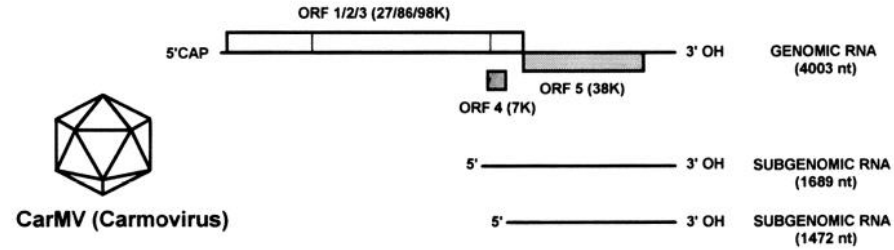



Fig. 1. Virus structure and genomic organization of carnation mottle virus (CarMV). Location of the five open reading frames is indicated, together with the sizes of the proteins they code for. Location and length of the two 3' coterminal subgenomic RNAs is also indicated.

coat protein (CP) is the 3'-terminal p38 reading frame. Two subgenomic RNAs (sgRNA) of 1.5 and 1.7 kb, coterminal with the 3' genomic end, are synthesized and encapsidated in vivo. In vitro translation assays have identified all the proteins that CarMV codes for ***(3–5)***. The most abundant translation products from the genomic RNA are p27 and p86, CP and p7 being almost silent ***(3)***. p98 was identified as an in vitro translation product of CarMV RNA only after using a lysate optimized for double readthrough ***(4)***, although comparison with other carmoviruses (*see next paragraph*) has questioned its in vivo existence. The smaller of the sgRNAs directs the translation of the CP p38; translation of the 1.7-kb sgRNA renders p7 ***(5)***.

TCV genome organization is closely related to that of CarMV ***(6)***. Remarkable differences are, however, that TCV lacks the second readthrough protein that would correspond to CarMV p98 and codes for a set of two, instead of one, small proteins (p8 and p9) in the central region of the genome. The use of infectious in vitro transcripts has allowed the identification of some of the functions of the TCV proteins ***(7)***. Thus, p8, p9, and the CP were shown to be required for systemic invasion; p28 and p88, which correspond to CarMV p27 and p86, were required for viral RNA replication.

In general, carmoviruses reach high levels of accumulation in their corresponding hosts. The virions are highly stable in plant sap, where they may remain infectious for long periods (2–6 wk) at room temperature. Ultrastructural studies have shown that the virus particles are easily recognized in the cytoplasm of infected cells. Carmoviruses are also good immunogens and generally there is not crossreactivity among them.

Initially, carmovirus purification methods consisted of the extraction of the tissue in neutral phosphate buffers and subsequent clarification with organic solvents, followed by differential and density gradient centrifugations ***(1)***.

Nelson and Tremaine *(8)* developed an alternative protocol in which the tissue was homogenized in an acidic sodium acetate buffer to achieve clarification of the extract in the absence of organic solvents. A later improvement of this method included a polyethylene glycol precipitation step *(9)* and has been widely used for carmovirus purification (*see* **Note 6**) Here, we describe a different modification of the acetate buffer method that renders high yields of high-quality virus in a significantly shorter time. The method described here is based on a high-speed centrifugation to pellet the virions through a sucrose cushion. Our experience with several carmoviruses shows that the virions obtained in this way are pure enough for different virological and molecular analyses (*see* **Notes 4** and **5**) Viral RNA extraction from purified virions is carried out essentially as described previously *(10)*.

## 2. Materials

All the solutions should be prepared with MilliQ water.

### 2.1. Virus Purification

1 Liquid nitrogen
2 0.2*M* Sodium acetate, pH 5 0 Autoclave and store at room temperature
3 10 m*M* Tris-HCl, pH 7 3 Autoclave and store at room temperature
4 20% (w/v) Sucrose in 10 m*M* Tris-HCl, pH 7 3 Freshly prepared in each experiment Alternatively, the solution can be autoclaved at 121°C for no more than 15 min to avoid caramelization of sucrose

### 2.2. Viral RNA Extraction

1 4 mg/mL Proteinase K dissolved in sterile water Store aliquoted at –20°C
2 10% (w/v) Sodium dodecyl sulfate (SDS) in 50 m*M* Tris-HCl, pH 8 0 Autoclave and store at room temperature
3 1*M* Tris-HCl, pH 7 6 Autoclave and store at room temperature.
4 5*M* NaCl Autoclave and store at room temperature
5. Phenol.chloroform:isoamyl alcohol (25 24.1). Prepared and stored as described by Sambrook et al *(11)* **Caution:** Phenol is highly toxic and gloves must be worn when handling it
6 Diethyl ether or chloroform **Caution:** Diethyl ether is highly volatile and should be stored and used in a fume hood
7 3*M* Sodium acetate, pH 5 5 Prepared and stored as described *(11)*
8 Absolute ethanol and 70% (v/v) ethanol in water Store at –20°C

## 3. Methods

When possible, keep the extract on ice or at 4°C. Also, once the virus purification (and RNA extraction) has been started, it should be carried out as quickly as possible. **Caution:** Caution must be taken with RNase contamination; gloves

should be worn and plasticware and glassware should be either autoclaved or treated with 15% $H_2O_2$ for 15 min, and then washed twice with autoclaved water.

### 3.1. Virus Purification

We normally extract *Chenopodium quinoa* leaves, since this is the experimental host for most of the viruses we work with In the case of CarMV and PFBV, the tissue is collected at 6–8 d postinoculation, which is about 2–3 d after the appearance of the first viral lesions. Tissue may be kept frozen for long periods at –20°C.

1 Homogenize the infected tissue to a fine powder in liquid nitrogen with mortar and pestle We use mortars for 5–20 g of tissue, when larger amounts are extracted, homogenization can be carried out in a food blender
2 Wait until the homogenized leaves defrost and then add 2 vol of 0 2*M* sodium acetate, pH 5 0 Keep mixing thoroughly for up to 5 min (*see* **Note 1**) Alternatively, the fine powder can be poured into a tube containing the buffer and mixed by vortexing
3 Filter the homogenate through cheesecloth to remove large debris
4 Centrifuge at 7700g in a Beckman JA.20 rotor for 20 min Keep the supernatant, which should be light green and free of any particulate material that would impair the next purification step
5 Slowly, layer the supernatant (10–15 mL) on top of 5 mL 20% sucrose in 10 m*M* Tris-HCl, pH 7 3, cushion, loaded in ultracentrifuge tubes Be careful to layer the sample on the center of the cushion surface, to avoid it sliding between the cushion and the walls of the tube Centrifuge at 146,000*g* in a Beckman 50 2 Ti rotor for 2 h
6 A small, lightly colored, and opalescent pellet should be visible Discard the supernatant and drain any excess by keeping the tube upside down on filter paper for a minute or so. Resuspend the pellet in 10 m*M* Tris-HCl, pH 7 3 (ca 0 04 of the initial volume) Make sure resuspension is complete. Resuspension will improve by pipeting in and out with an automatic pipet If needed, pellet can be left overnight with buffer at 4°C
7 Transfer to an Eppendorf tube Centrifuge at top speed for 1 min in an Eppendorf centrifuge to remove any unresuspended material
8. Keep the supernatant as purified virus fraction (*see* **Note 2**) Store aliquoted at –20°C In general, carmoviruses are rather stable, in our experience, aliquots of purified virus stored at –20°C retained infectivity for several months

### 3.2. Viral RNA Extraction

1. Incubate the purified virus at 37°C for 1 h in 400 µL of a solution containing 50 µg/mL proteinase K, 0.5% SDS, 10 m*M* Tris-HCl, pH 7 6, and 0 1*M* NaCl We have extracted up to 2 mg of virions in a single Eppendorf tube with good yields
2. Extract twice with 1 vol of phenol·chloroform isoamyl alcohol.

3 Extract twice with 1 vol of diethyl ether or chloroform.
4 Add 0 1 vol of 3*M* sodium acetate, pH 5 5, and 2 vol of cold absolute ethanol. Mix thoroughly and leave at –20°C for 2 h, or at –70°C for 30 min, to allow RNA precipitation
5 Collect the RNA by centrifugation for 15 min at top speed in an Eppendorf centrifuge at 4°C
6 Wash the pellet with 70% ethanol
7 Dry the pellet and resuspend it in sterile water (*see* **Note 3**)

## 4. Notes

1 Be aware that the pH of the homogenate does not become alkaline during extraction Some carmoviruses tend to swell by the shift from acidic to slightly basic pHs, making the viral RNA susceptible to the degradation by nucleases The use of low pH extraction buffers avoids this possibility, having the additional advantage that many of the host proteins precipitate at these pHs.
2 Measure the virus concentration by UV absorption (1 $OD_{260}$ is 5 mg/mL for CarMV) We routinely follow the procedure described here in the purification of PFBV and CarMV, with yields that range from 1 6–3 mg virus/100 g tissue for PFBV, up to 2 mg/g in the case of CarMV Purity can be checked with agarose gel-TBE electrophoresis *(11)*, direct electrophoresis of the virion solution will render a single ethidium bromide-stained band, when free of contaminants After ethidium bromide staining, the agarose gel is suitable for Coomassie blue staining to reveal proteins, again, a single band located at the same position should appear in reasonably pure preparations Additionally, protein contamination can be assayed with polyacrylamide gel electrophoresis *(12)*
3. Measure viral RNA concentration by UV absorption, to calculate RNA extraction yield, consider that carmovirus particles have a 17–22% RNA content *(1)* RNA quality and contamination can be checked by agarose gel-TBE electrophoresis *(11)*, a single sharp band should be visible; sometimes weak smaller bands corresponding to sgRNAs also appear In order to store the viral RNA for long periods, we strongly recommend addition of 0 1 vol of 3*M* sodium acetate, pH 5 5, and 2 vol of absolute ethanol to the RNA solution, keep in aliquots as ethanol precipitate at –70°C
4 In our experience, the virus preparation obtained by high-speed pelleting through sucrose cushions has excellent infectivity and is pure enough and suitable for several experimental approaches For instance, we have used viral RNA extracted from virions purified in this way for in vitro translation, Northern detection, cDNA synthesis to obtain nonradioactive probes, and direct RNA sequencing by reverse transcription
5 Should a higher purity of virions be needed, the method described above can be continued by centrifugation through a linear sucrose gradient as follows
   a Layer the virus fraction obtained in **Subheading 3.1., step 8** on a 10–40% sucrose in 10 m*M* Tris-HCl, pH 7.3, gradient. We recommend preparation of the gradient the day before by loading in ultracentrifuge tubes equal volumes

of 40, 30, 20, and 10% sucrose in 10 m*M* Tris-HCl, pH 7 3, let it diffuse at 4°C overnight. Centrifuge at 82,000*g* in a Beckman SW40 rotor for 2 h

b Remove equal volume fractions and analyze them by UV absorption and/or agarose electrophoresis (*see* **Note 2**)

c. Pool together fractions containing the virus, dilute them twofold with 10 m*M* Tris-HCl, pH 7 3, and centrifuge at 146,000*g* in a Beckman 50 2 Ti rotor for 2 h.

d. Resuspend the pellet in 10 m*M* Tris-HCl, pH 7 3 Remove unresuspended material as in **Subheading 3.1., step 7**

6 There is an alternative method to the one described here that includes a polyethylene glycol (PEG 6000) precipitation step *(9)* If followed, supernatant obtained in **Subheading 3.1., step 4** is brought to 8% PEG 6000 and 200 m*M* NaCl, and stirred for 1 h at 0–4°C Virions are then pelleted by centrifugation at 7700*g* for 20 min Pellets are resuspended in 10 m*M* Tris-HCl, pH 7 3, and solution is finally ultracentrifuged at 146,000*g* for 2 h to pellet the virions again We must stress, however, that in side-by-side experiments the sucrose cushion protocol described here rendered higher amounts (as much as twice) of better quality virus preparations, in a significantly shorter period of time, when compared to the PEG precipitation protocol

7 As reported previously *(1)*, caution must be taken with carmoviruses that have been reported to be isoelectric at acidic pHs, since virus precipitation may occur during extraction In these cases, tissue must be extracted in neutral phosphate buffers and clarification of the homogenate achieved with organic solvents (*n*-butanol and/or chloroform) *(1)* We do not predict any problems with the use of centrifugation through sucrose cushions in these examples

## References

1 Morris, T J and Carrington, J C (1988) Carnation mottle virus and viruses with similar properties, in *The Plant Viruses, vol. 3. Polyhedral Virions with Monopartite RNA Genomes* (Koenig, R , ed ), Plenum, New York, pp 73–112

2 Guilley, H , Carrington, J C , Balàzs, E , Jonard, G., Richards, K., and Morris, T J. (1985) Nucleotide sequence and genome organization of carnation mottle virus RNA *Nucleic Acids Res* **13,** 6663–6677

3 Carrington, J C and Morris, T J (1985) Characterization of the cell-free translation products of carnation mottle genomic and subgenomic RNAs *Virology* **144,** 1–10.

4 Harbison, S A , Davies, J W , and Wilson, T M A (1985) Expression of high molecular weight polypeptides by carnation mottle virus RNA *J Gen Virol* **66,** 2597–2604

5 Carrington, J. C and Morris, T J (1986) High resolution mapping of carnation mottle virus- associated RNAs *Virology* **150,** 196–206

6 Carrington, J C , Heaton, L A , Zuidema, D , Hillman, B I , and Morris, T J. (1989) The genome structure of turnip crinkle virus *Virology* **170,** 219–226

7 Hacker, D L , Petty, I T D , Wei , N , and Morris, T J (1992) Turnip crinkle virus genes required for RNA replication and virus movement *Virology* **186,** 1–8

8 Nelson, M R and Tremaine, J H. (1975) Physicochemical and serological properties of a virus from saguaro cactus *Virology* **65,** 309–319
9 Lommel, S A , McCain, A H , and Morris, T J (1982) Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses *Phytopathology* **72,** 1018–1022
10 Carrington, J C and Morris, T J (1984) Complementary DNA cloning and analysis of carnation mottle virus RNA. *Virology* **139,** 22–31
11 Sambrook, J , Fritsch, E F , and Maniatis, T (1989) *Molecular Cloning A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
12. Laemmli, U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature (London)* **227,** 680–685

# 22

# Tymovirus Isolation and Genomic RNA Extraction

**Adrian Gibbs and Anne M. Mackenzie**

## 1. Introduction

At least 20 tymovirus species are known. The generic acronym, tymovirus, comes from *t*urnip *y*ellow *mo*saic virus (TYMV), the name of the first to be described *(1)*, now the type species The tymoviruses infect dicotyledonous angiosperms, mostly those that have the C3 photosynthetic pathway; few have been recorded in crop plants, and none are known to infect monocotyledonous angiosperms. They cause bright yellow mosaics, vein-clearing, and mottling. Their natural vectors, when known, are beetles, mostly halticid or galerucid flea beetles Only four tymoviruses have been recorded as seed-borne *(2)*

Tymoviruses have been recorded from all continents except Antarctica. Most species have only been recorded from a single continent and often from only a single noncrop plant species, after which they are named; cacao yellow mosaic, okra mosaic, and voandezia necrotic mosaic viruses from Africa; abelia latent, desmodium yellow mottle, eggplant mosaic, passiflora yellow mosaic, physalis mosaic (syn. BMV-Iowa), plantago mottle, potato Andean latent, and wild cucumber mosaic viruses from the Americas; kennedya yellow mosaic virus from Australia; belladonna mottle, dulcamara mottle, erysimum latent, ononis yellow mosaic, and scrophularia mottle viruses from Europe; melon rugose mosaic virus from the Middle East, turnip yellow mosaic from Australia and Europe, clitoria yellow vein from Africa and Southeast Asia, and poinsettia mosaic virus, which is only tentatively grouped with the tymoviruses, is found in ornamental poinsettias worldwide

The serological relationships of tymovirus virions correlate with their known genomic sequence differences and place them in four groups: the turnip yellow mosaic viruses, the legume-infecting species plus cacao yellow mosaic and okra mosaic, those infecting solanaceous plants plus ononis yellow mosaic and

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

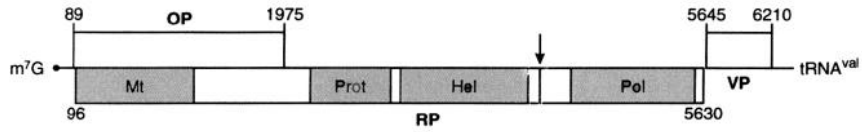



Fig. 1. Map of the 6319-nucleotide long genome of turnip yellow mosaic tymovirus. The first and last nucleotides of the ORFs of the replicase polyprotein (RP), overlapping protein (OP), and virion protein (VP) are numbered, and the portions of the RP that probably function as a methyltransferase (Mt), papain-like protease (Prot), NTPase/helicase (Hel), and RNA-dependent RNA polymerase (Pol) are shaded. The arrow marks the nucleotides encoding the site, between $ala^{1259}$ and $thr^{1260}$, where the RP is posttranslationally hydrolyzed.

wild cucumber mosaic, and finally erysimum latent, which is the only member of a fourth lineage.

Tymoviruses cause yellow mosaic and vein-clearing symptoms by clumping and disorganizing the chloroplasts of infected cells *(3)*. The chloroplasts develop characteristic patches of peripheral vesicles, especially where they touch. The vesicles develop as invaginations of the outer chloroplast membranes and remain attached by their necks. The vesicles are the site of viral genomic replication, but the subgenomic virion protein mRNA, at least, is translated by cytoplasmic ribosomes, and the virions assemble on, or near, the cytoplasmic end of the vesicle necks.

The virions of tymoviruses are isometric, about 28 nm in diameter, and have a shell that is a regular T = 3 icosahedron constructed of 180 subunits of a single protein species. Virions sediment at 110–120 S and each contains a single viral genome, which constitutes 35% of their mass. All tymoviruses also produce virion-like particles that sediment at 50–55 S and consist of genome-free protein shells. The virions and/or the empty shells of some tymoviruses also contain small RNA molecules: virion protein (VP) mRNA or host tRNAs.

The genomes of all tymoviruses are single-stranded RNA about 6.3 kb in length, and are infectious when chemically separated from the virions. They have an unusally large cytosine content, up to 42% on average, and even more in the third codon positions of the replicase and virion protein genes. The genomes of all tymoviruses have three open reading frames (ORFs) (**Fig. 1**). There are small untranslated regions at both termini, and also, in some, between the largest and smallest of the ORFs; the 3'-terminus of most can form a tRNA-like structure that can be specifically valylated.

The largest of the ORFs is the most conserved and occupies most of the genome. It encodes the replicase protein (RP) of approx 206 kDa, which has motifs (N- to C-terminal) characteristic of a *N*-methyl transferase *(4)*, a papain-

like protease *(5,6)*, a NTPase/helicase *(7)*, and a RNA replicase *(8)*. After translation, the RP is hydrolyzed specifically by the protease to yield a 66-kDa C-terminal fragment containing the replicase, and a 140-kDa fragment *(9)*.

Overlapping the 5'-terminal third of the RP, and always starting seven nucleotides to the 5' side of its start codon, is the least conserved ORF. It encodes the overlapping protein (OP) of approx 69 kDa that is very basic (pI 10.9–11.9).

The third and smallest ORF is between the RP gene and the 3' end of the genome It encodes the virion protein (VP; 188–202 amino acid residues) of approx 20 kDa, and is expressed via a subgenomic RNA. A region of about 50 nucleotides to the 5' side of the start of the VP ORF, and including the 3' terminus of the VP ORF, has a closely similar sequence in all tymoviruses One part of it, the tymobox, is probably the complement of the transcription promoter sequence of the VP gene, and is identical in eleven tymoviruses (5'-dGAGTCTGAATTGCTTC-3'), with a single difference in another three, and four differences in that of the wild cucumber mosaic virus genome (5'-dGAGTCTTCTTTGCATC-3') *(10)*. An oligonucleotide with the tymobox sequence is thus useful as a probe for tymoviruses or as a PCR primer for isolating the virion protein gene of most tymoviruses.

## 2. Materials

1 Infected plant tissue Tymoviruses are readily transmitted by manual inoculation, and their virions attain large concentrations in infected plants TYMV infects a wide range of brassicas, but its virions attain very large concentrations (0 5–2 0 mg/g leaf tissue) in young plants of Chinese cabbage (*Brassica campestris* ssp *chinensis* [Pak-choi] and ssp. *pekinensis* [Pe-tsai]) grown in rich, frequently watered compost, in bright light of 12–16 h/d, and a daytime maximum temperature of 25°C The plants are best inoculated at the 3–6 leaf stage, and inoculated and systemically infected leaves harvested 2–4 wk later

2 PA buffer: 100 m*M* $Na_2HPO_4$, 50 m*M* ascorbic acid, pH 7 0

3. 50·50 chloroform *n*-butanol a 50.50 v/v mixture of chloroform and *n*-butanol

4. TE buffer 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7 0

5 SSC 150 m*M* NaCl, 15 m*M* sodium citrate

6 RNA extraction buffer 10 m*M* KCl, 1 5 m*M* $MgCl_2$, 0 2% sodium dodecyl sulfate (SDS) in 10 m*M* Tris-HCl, pH 7.4.

7 TE-saturated phenol· phenol equilibrated with about 0 5 vol TE buffer by shaking them together several times, and then keeping the mixture overnight at 4°C for the phases to separate

## 3. Methods

### *3.1. Virion Purification*

Tymovirus virions are readily purified by a wide range of methods, because they are stable and attain large concentrations in the tissues of infected host

plants, thus, tymoviruses are ideal for teaching virological techniques, and, unlike tobamoviruses, are not contagious, although they are readily sap transmitted

The earliest methods used to purify the virions of TYMV exploited differences in the solubility and stability of the virions and the host sap constituents, and used only low *g*-force centrifuges. The best of these methods were the ethanol:ammonium sulfate method *(1)* and the pH 4.8 method *(11)*, although we have found the latter to be unreliable

We find that variants of Steere's method *(12)* work well for all the 15 or so tymoviruses that we have studied. This method uses organic solvents and differential high-speed centrifugation

1 Blend fresh or frozen infected leaves in 1 5-4 vol/wt of PA buffer
2 Blend further while slowly adding 0 25 vol of 50 50 chloroform *n*-butanol to form an emulsion
3 Centrifuge the emulsion at 5000*g*, collect, and filter the aqueous (top) layer through a small cotton wool plug
4 Concentrate and purify the virions by one cycle, or more, of differential centrifugation (100,000*g* for 3 h, and 5000*g* for 10 min), using 50 m*M* phosphate buffer, pH 7 0–8 0, for resuspending the virions
5 The virions can be further purified and separated from the shells by centrifuging in sucrose gradients (10–40% sucrose in TE buffer at 113,000*g* for 2 h) or in cesium chloride gradients
6 The final virion preparation is usually dialyzed into SSC for use as a source of genomic RNA, or for use as an immunogen If not used immediately virion preparations may be stored at 4°C, but it is best to add sodium azide to give a concentration of 1–5 m*M*

The final suspension of virions (2 mg/mL obtained from each 10 g tissue) can be used for preparing crystals

### 3.2. Genomic RNA Preparation

We prepare TYMV genomic RNA from purified virions using a proteinase K method *(14)*

1. Mix 1 vol of virion preparation in SSC with 4 vol of RNA extraction buffer
2 Add proteinase K to give a concentration of 0 8 mg/mL and incubate at 56°C for 15 min
3 Add 1*M* NaCl to restore its concentration to 0.15*M*
4 Add 1 vol of TE-saturated phenol and mix thoroughly
5. Add 1 vol of chloroform, mix thoroughly, then centrifuge at 5000*g* for 10 min and collect the aqueous (upper) phase carefully
6. Repeat **steps 4** and **5** above
7. Precipitate the RNA by adding 0.1 vol 3*M* sodium acetate, pH 5 2, and 2 5 vol ethanol, mix, place at –20°C for 15 min, and centrifuge at 15,000*g* for 10 min

8. Wash the pellet with cold 70% ethanol and dry.
9. Resuspend the precipitated RNA in TE buffer and store at –20°C

## 4. Notes

1 All the buffers used in the methods described above are stable, except for the phosphate ascorbate buffer used for Steere's method. It is best made from the dry chemicals just before use, dissolve the ascorbic acid first, because it rapidly degrades above pH 8.0 and produces a yellow compound

2. **Caution:** Take care with the fumes of chloroform and *n*-butanol, use these in a fume hood or well-ventilated space because chloroform is an anesthetic and *n*-butanol causes breathing difficulties

3 The centrifugation times and *g*-values given above are only indicative and depend on the machinery available When sedimenting the virions by centrifugation alone, be guided by the fact that long centrifugation times at large *g*-values may produce pellets that are difficult to resuspend, whereas smaller *g*-values and insufficient centrifugation times will produce unstable pellets depleted in virion-like protein shells

4 We have found the methods described above to work well for more than a dozen tymoviruses, we always use PA buffer for extraction when the propagation host of the tymovirus produces a sap extract that oxidizes and becomes brown (e g , *Nicotiana glutinosa* with eggplant mosaic virus) However, for other hosts, TE buffer is an alternative to PA buffer for all steps, and, for larger preparations, the first high-*g* centrifugation step can be replaced by adding 4% NaCl (w/v) and 12% w/v polyethylene glycol (mol wt 8000), stirring for 1 h, and collecting the sediment by centrifuging at 5–10,000*g* for 20 min

5 The pI of TYMV virions is 3.75, and those of cacao, kennedya, and ononis yellow mosaic viruses are similar, those of belladonna mottle, dulcamara mottle, and eggplant mosaic viruses have pIs above 8 0 (J -K Mo, M Fischer and A Gibbs, unpublished results); however, we obtained no better preparations of the basic virions using buffers of pH 5.0

6 An alternative method that clearly produces very pure virion preparations uses ethanol and centrifugation to prepare TYMV virions for X-ray diffraction analysis ***(13)***, 20 m*M* potassium phosphate buffer, pH 7.8, and a temperature of 4°C, is used for all stages of the procedure·

   a Blend infected leaves in 1 5 vol/wt of buffer
   b Filter the fiber from the buffered extract using cheesecloth
   c Add 0 25 vol of 95% ethanol, stir for 30 min
   d Remove the sediment by centrifuging for 10 min at 5000*g*
   e Centrifuge the supernatant at 104,000*g* for 1 5 h to sediment and concentrate the virions
   f Resuspend the virions in buffer; clarify the preparation by centrifuging for 10 min at 5000*g*
   g Further purify the virions by another round of centrifugation, 186,000*g* for 1 5 h, 5000*g* for 10 min

7 The virion protein gene of tymoviruses may be amplified using the tymobox primer The 3'-terminus of genomic RNA is polyadenylated by standard methods, then a synthetic dTnG primer (usually $n = 8$) used to prime synthesis of first strand cDNA The virion protein gene can then be specifically amplified by the polymerase chain reaction using a dTnG primer and the tymobox primer The resulting fragment, which is ca 700 bp in size, can be cloned into the *Sma*I site of M13mp18 or the *Eco*RV site of Bluescript SKT for sequencing

## References


1 Markham, R. and Smith, K. M. (1949) Studies on the virus of turnip yellow mosaic *Parasitology* **39,** 330–342

2 Brunt, A , Crabtree, K , Dallwitz, M , Gibbs, A , and Watson, L (1996) *Viruses of Plants* CAB International, Wallingford, UK

3 Matthews, R E F (1991) *Plant Virology*, 3rd ed Academic, New York, pp 231–239, 404–406

4 Rozanov, M N , Koonin, E V , and Gorbalenya, A E (1992) Conservation of the putative methyltransferase domain a hallmark of the "Sindbis-like" supergroup of positive-strand RNA viruses *J Gen Virol* **73,** 2129–2134

5 Bransom, K L and Dreher, T W (1994) Identification of the essential cysteine and histidine residues of the turnip yellow mosaic virus protease *Virology* **198,** 148–154

6 Rozanov, M N , Drugeon, G , and Haenni, A -L (1995) Papain-like proteinase of turnip yellow mosaic virus a prototype of a new viral proteinase group *Arch Virol* **140,** 273–288

7. Gorbalenya, A. E. and Koonin, E. V. (1989) Viral proteins containing the purine NTP-binding sequence pattern *Nucleic Acid Res* **17,** 8413–8440

8 Kamer,G and Argos, P (1984) Primary structural comparion of RNA-dependent polymerases from plant, animal and bacterial viruses *Nucleic Acid Res* **12,** 7269–7282

9 Kadaré, G., Rozanov, M , and Haenni, A.-L. (1995) Expression of the turnip yellow mosaic virus proteinase in *Escherichia coli* and determination of the cleavage site within the 206 kDa protein *J Gen Virol* **76,** 2853–2857

10 Ding, S , Howe, J , Keese, P , Mackenzie, A , Meek, D , Osorio-Keese, M , Skotnicki, M , Srifah, P , Torronen, M , and Gibbs, A (1990) The tymobox, a sequence shared by most tymoviruses its use in molecular studies of tymoviruses *Nucleic Acid Res* **18,** 1181–1187

11 Matthews, R E F (1960) Properties of nucleoprotein fractions isolated from turnip yellow mosaic virus preparations *Virology* **12,** 521–539

12 Steere, R L. (1956) Purification and properties of tobacco ringspot virus *Phytopathology* **46,** 60–69

13 Canady, M A., Day, J , and McPherson, A (1995) Preliminary X-ray diffraction analysis of crystals of turnip yellow mosaic virus (TYMV). *Proteins* **21,** 78–81

14 Both, G W and Air, G M (1979) Nucleotide sequence for the N-terminal region of the matrix protein of influenza virus *Eur J Biochem* **96,** 363–372

# 23

# Tombusvirus Isolation and RNA Extraction

**József Burgyán and Marcello Russo**

## 1. Introduction

The group Tombusvirus is one of the 16 groups of plant viruses established in 1971 *(1)*. Its name derives from the sigla "tombus" from *tom*ato *bu*shy *s*tunt, which is the disease caused by the type member of the group, tomato bushy stunt virus (TBSV). The group was later ranked as genus *Tombusvirus* in the family *Tombusviridae*, which includes also the genus *Carmovirus*, in the new classification of plant viruses in families, genera, and species approved by the International Committee on Taxonomy of Viruses (ITCV) at the Ninth International Congress of Virology in Glasgow in 1993 *(2)*. A list of selected definitive members of the genus is reported in **Table 1**.

The natural host range of individual virus species is rather narrow and restricted to dicotyledons, infecting very few or only one host. But the artificial host range is wide and is comprised of several plant species in 20 different dicotyledonous and monocotyledonous families *(3)*.

The majority of the tombusvirus species occur in temperate regions, where they occasionally cause outbreaks with economic relevance. Diseases of major importance have been reported for tomato, pepper, and eggplant caused by TBSV, for artichoke caused by artichoke mottled crinkle (AMCV), for eggplant caused by eggplant mottled crinkle (EMCV), for pepper and tomato caused by Moroccan pepper (MPV), and for cherry caused by petunia asteroid mosaic (PAMV) *(3)*. Tombusviruses have stable, highly infectious particles, which are readily transmissible mechanically, they are often found in natural environments such as soil and surface water, from which they can be acquired by the hosts in the absence of respective hosts without the assistance of vectors. For only cucumber necrosis virus has it been demonstrated that soil-transmission is favored by the soil inhabiting chytrid fungus *Olpidium bornovanus (4,5)*.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

**Table 1**
**Selected Species in the Genus *Tombusvirus*[a]**

| |
|---|
| Artichoke mottled crinkle (AMCV) |
| Carnation Italian ringspot (CIRV) |
| Cucumber necrosis (CNV) |
| Cymbidium ringspot (CymRSV) |
| Eggplant mottled crinkle (EMCV) |
| Moroccan pepper (MPV) |
| Pelargonium leaf curl (PLCV) |
| Petunia asteroid mosaic (PAMV) |
| Tomato bushy stunt (TBSV) |

[a]*See* **ref. *8*** for complete list and references

Tombusviruses have isometric particles ca 30 nm in diameter, with a somewhat rounded outline and a surface structure poorly resolved in the electron microscope. Virions are T = 3 icosahedra consisting of 180 identical structural subunits, clustered in dimers to give rise to 90 morphological units. The structural subunit is folded into distinct domains: R, the N-terminal internal domain interacting with RNA, a, a connecting arm; S, the shell domain constituting the capsid backbone; and P, the C-terminal domain connected by a short hinge to the S domain and protruding in pairs from the particle surface to form 90 projections *(6)*. The P domain determines the immunological and other biological properties of the virions.

Tombusvirus genome is constituted by a linear single-stranded monopartite RNA molecule of positive-sense ca 4700 nucleotides (nt) long, which contains five open reading frames (ORF) coding for proteins with an approximate mol wt of 33, 92, 41, 22, and 19 kDa (**Fig. 1**). Translation products of ORFs 1 and 2 are expressed by genomic-length viral RNA; ORF 3, and ORFs 4 and 5 are expressed through two 3' coterminal subgenomic RNAs of ca. 2.2 and 1 0 kb, respectively. The readthrough domain of ORF 2 is the viral RNA-dependent RNA polymerase, because it contains the eight conserved motifs (PI–PVIII) that characterize the RNA polymerase of supergroup II of positive-sense RNA viruses *(7)* The product of ORF 3 is the capsid protein The protein encoded by ORF 4 is the movement protein involved in the cell-to-cell spread of virus in infected tissues. The functions of translation products of ORFs 1 and 5 are not yet established with certainty; however, circumstantial evidence suggests that the product of ORF 1 may be responsible for intracellular localization of the viral replicative structures and that of ORF 5 carries determinants affecting severity of symptoms. Artificial viral mutants that cannot express ORF 5 are still infectious, but induce milder symptoms, compared to wild-type Conversely, the presence of ORF 1 is an absolute prerequisite for viral viability *(8)*.

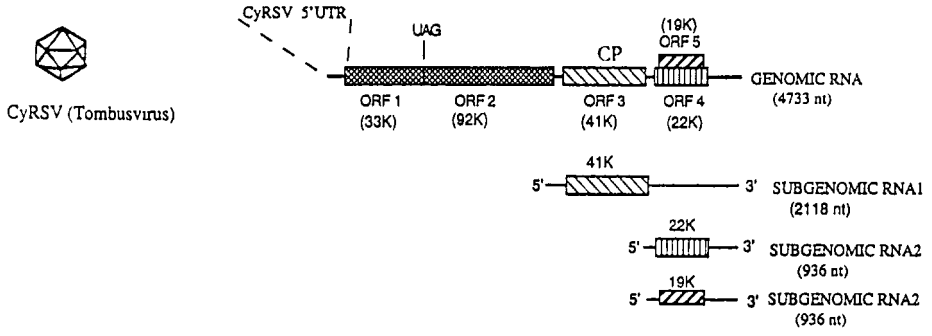



Fig 1 Virus structure, genome organization, and expression strategy of a tombusvirus (CyRSV) Noncoding regions are shown as solid lines (UTR = untranslated leader sequence) and ORFs by boxes with different shading The approximate mol wt of predicted translation products, and sizes and locations of subgenomic RNAs, are indicated The virus CP (41 kDa) is encoded by ORF 3

Tombusviruses may support the replication of two types of subviral RNAs: satellite and defective-interfering (DI) RNAs Satellite RNA is a linear molecule of 619 nt, with little sequence in common with the genomic RNA, DI RNAs (400–800 nt) are deletion mutants of viral genomes that have generally lost all essential viral genes required for movement, replication, and encapsidation ***(9)*** Both satellite and DI RNAs require the presence of a helper virus for *trans*-acting factors necessary for replication ***(8)***.

## 2. Materials

### 2.1. Virus Purification

1 0 5*M* sodium acetate buffer, pH 5 5 (stock solution)
2. Polyethylene glycol (PEG) 6000
3 NaCl
4 Homogenization buffer 0 1*M* sodium acetate buffer, pH 5 5, containing 0 25% β-mercaptoethanol (freshly prepared)
5 High-speed (15K rpm) homogenizer
6 Beckman J2-21 low-speed centrifuge, with rotor JA-20 or equivalent.
7 Beckman L7-55 ultracentrifuge, with rotor Type 40 or equivalent
8 Eppendorf microcentrifuge, or equivalent

### 2.2. RNA Extraction

1. RNA extraction buffer 0 1*M* glycine-NaOH, pH 9.0, containing 100 m*M* NaCl, 10 m*M* EDTA, 2% sodium dodecyl sulfate (SDS), and 1% sodium lauroyl sarcosine
2 3*M* Sodium acetate, pH 5 5

3 Water-saturated phenol, containing 0 1% 8-hydroxyquinoline
4 Chloroform
5 Ethanol 100 and 70%, autoclaved water

**Caution:** Take care in handling organic solvents (use gloves) and avoid breathing vapors.

## 3. Methods

### 3.1. Virus Purification

1 Collect infected leaves of *Nicotiana benthamiana* or *N clevelandii* 10–14 d after inoculation, and homogenize them in cold homogenization buffer, 1 g tissue/3 mL of buffer (*see* **Notes 1** and **2**)
2 Squeeze through cheesecloth
3 Leave the extract in ice for 30 min Most plant proteins will precipitate because of the low pH
4. Clarify by low speed centrifugation (12,000*g* for 10 min) (*see* **Note 3**)
5 Transfer the supernatant (where the virus particles are) into a beaker, and adjust to pH 6 0 with dilute NaOH
6. Add 10 g PEG and 1.1 g NaCl/100 mL and dissolve with magnetic stirrer. Keep the solution on ice for 1 h
7 Precipitate the virus particles by low-speed centrifugation (12,000*g* for 10 min) and discard the supernatant
8 Resuspend the pellet in 0 02*M* sodium acetate, pH 5 5 (use a vortex), leave in ice for 1 h and vortex again
9 Clarify the virus solution by low-speed centrifugation (12,000*g* for 10 min) Save the supernatant
10 Sediment virus particles by high-speed centrifugation (90,000*g* for 1 h), discard the supernatant
11 Let the pellet dissolve in 0 02*M* sodium acetate, pH 5 5, for several hours or overnight at 4°C Vortex to dissolve completely the virus pellet, transfer to an Eppendorf tube, and eliminate the insoluble material by low-speed centrifugation in an Eppendorf centrifuge

This virus preparation is now sufficently pure for RNA extraction. However, if highly purified virus is needed, further purification can be achieved through density gradient centrifugation in CsCl at equilibrium To do so, continue as follows:

12 Dissolve 2 65 g of CsCl in 5 mL virus suspension (initial density of CsCl solution is 1 36 g/mL) and centrifuge at 90,000*g* for 16 h at 10°C
13 Collect the sharp opalescent virus band by puncturing the tube with a syringe and removing the band Remove the CsCl by dialysis against 50 m*M* NaCl, pH 5 5

Virus yield ranges between 10 and 60 mg/100 g of infected tissues. The virus preparation can be stored at –70°C.

### 3.2. RNA Extraction from Virus Particles

1 Add 1 vol of RNA extraction buffer and 2 vol water-saturated phenol to virus solution, vortex for 30–60 s, and centrifuge at maximum speed in microcentrifuge for 5 min at room temperature
2 Transfer the aqueous phase (upper) to a fresh tube, extract once with equal volumes of phenol.chloroform, and centrifuge for 5 min
3 Extract the aqueous phase again with chloroform, and centrifuge for 2 min
4 Transfer the aqueous phase to a fresh tube, add 2 5 vol of cold ethanol and 0 10 vol of 3*M* sodium acetate, pH 5 5, mix well, and centrifuge for 10 min at 4°C in a microcentrifuge
5 Wash the RNA pellet with 70% cold (–20°C) ethanol, dry, and resuspend the RNA in ice-cold sterile water Store at –70°C for long periods

### 3.3. Total RNA Extraction from Leaf Tissue

1 Precool mortar and pestle on ice for several minutes
2. Rapidly homogenize 50–100 mg infected leaf tissue in ice-cold mortar with pestle, and immediately add 600 µL RNA extraction buffer
3 Rapidly transfer the mix to a microtube containing an equal volume of phenol, vortex for 30–60 s and centrifuge at maximum speed in microcentrifuge for 5 min at room temperature
4. Proceed as above from **step 2**

### 3.4. Total RNA Extraction from Protoplasts

Total RNA can be extracted in a similar way from protoplasts. Discard the incubation medium and disrupt the protoplasts (approx $1–2 \times 10^6$) in 600 µL RNA extraction buffer, add an equal volume of phenol, and proceed following the same protocol described above.

## 4. Notes

1 *N benthamiana* or *N clevelandii* plants, normally used to propagate tombusviruses, can be inoculated with infected plant sap, purified virus or viral RNA The protocol described in **Subheading 3.1.** is applicable to all tombusviruses and gives consistently reproducible results.
2 To obtain good virus yield, it is important to used a high speed homogenizer The homogenization buffer must be prepare fresh The low pH is important for the stability of virus particles, extraction media at pHs above neutrality are detrimental to tombusvirus particles
3. The clarified sap (after **Subheading 3.1., step 4**) must be pale yellow If plants are harvested too old and/or necrotic, the virus pellet may result in contamination by some dark material and may be difficult to clean further
4 It is important to resuspend the virus pellet completely, especially after PEG precipitation, otherwise, much can be lost

5 To obtain a good RNA preparation, it is essential to avoid contamination with RNases. Laboratory glassware should be treated by baking at 180°C for 3 h or more It is best to use sterile disposable plasticware (Eppendorf microtubes, tips, pipets, and so on) whenever possible, since it is essentially free of RNases A potential major source of contamination with RNase are the hands of the investigator; disposable gloves should be worn during the RNA manipulation All solutions should be prepared using RNase-free glassware, autoclaved water, and chemicals reserved for work with RNA that should be handled with baked spatulas

6 The concentration of RNA can be determined spectrophotometrically by reading at wavelength of 260 nm An $OD_{260}$ = 1 corresponds to approx 40 µg of RNA/mL To estimate concentration in a sample, prepare a dilution 1 25 in water and read at 260 nm; the reading will directly give the concentration of RNA in mg/mL

## References

1 Harrison, B D , Finch, J T , Gibbs, A J , Hollings, M , Shepherd, R I , Valenta, R J , and Wetter, C (1971) Sixteen groups of plant viruses *Virology* **45,** 356–363

2 Martelli, G P and Russo, M (1994) Tombusviridae, in *Classification and Nomenclature of Viruses 6th Report of the International Committee on Taxonomy of Viruses* (Fauquet, C M , Murphy, F , Bishop, D H L , Summers, M D , Martelli, G P, Ghabrial, S A., and Jarvis, A W , eds ), (*Arch Virol* **Suppl. 10**)

3 Martelli , G P, Gallitelli, D , and Russo, M (1988) Tombusviruses, in *The Plant Viruses Polyhedral Virions with Monopartite RNA Genomes*, vol 3 (Koenig, R , ed ), Plenum, New York, pp 13–72

4 Dias, H F (1970) Transmission of cucumber necrosis virus by Olpidium cucurbitacearum Barr and Dias *Virology* **40,** 828–839

5 Stobbs, L W , Cross, G W , and Manoch, M S (1982) Specificity and methods of transmission of cucumber necrosis virus by Olpidium radicale zoospores. *Can J Plant Pathol* **4,** 134–142

6 Harrison, S C. (1983) Virus Structure. high resolution perspectives *Adv Virus Res* **28,** 187–240

7 Koonin, E. V. (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses *J Gen Virol* **72,** 2197–2206

8 Russo, M., Burgyan, J , and Martelli, G. P (1994) Molecular biology of Tombusviridae *Adv Virus Res* **44,** 381–428.

9 Roux, L , Simon, A E , and Holland, J J (1991) Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo *Adv Virus Res* **40,** 181–211

# 25

# RNA Analysis

## *Size and 3' End Group Determination*

**Michael Shanks and George Lomonossoff**

## 1. Introduction

The most commonly used method to analyze the quality of RNA is electrophoresis in agarose gels The distance an RNA molecule moves in a gel is dependent both on its mol wt and its conformation. Hence, to accurately compare RNAs across virus groups or to determine their size, it is crucial to completely denature the sample. Formaldehyde was the first denaturant to be used for this purpose *(1)* and is still a popular choice. Other reagents that denature but do not degrade RNA include formamide, which destroys base pairing, and glyoxal (ethanedial). Glyoxalation introduces an additional ring into guanosine residues, which then sterically hinders GC-pair formation. The first method described here incorporates formaldehyde in the gel and is adapted from Lehrach et al. *(2)*. The second approach, which is based on the method of McMaster and Carmichael *(3)*, uses glyoxal to denature the sample prior to loading it onto a nondenaturing gel The latter technique has the advantage of allowing native (nondenatured) RNA to be run on the same gel as denatured RNA

The last part of this chapter deals with the identification of the 3'-terminal base of an RNA molecule This is important, since many cloning techniques result in the addition of extra (nonviral) nucleotides at the 3' end of the viral sequence. It may, therefore, not be possible to deduce the true 3' end of a viral RNA by sequence analysis of cloned cDNA The technique employed makes use of T4 RNA ligase to add cytidine 3',5'-*bis*(phosphate) (pCp) to the 3'-OH terminus of the RNA of interest. By using [5'-$^{32}$P]pCp, the product of the reaction has a $^{32}$P phosphate group in the last phosphodiester linkage, effectively labeling the RNA at its 3' terminus *(4)*. Complete alkaline hydrolysis of the

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

RNA releases nucleoside 3' monophosphates, resulting in the transfer of the $^{32}P$ phosphate group to the nucleotide originally at the 3' end of the RNA. This nucleotide can then be identified by its electrophoretic mobility on paper, allowing the identity of the 3'-terminal nucleotide to be deduced. The 3'-labeled RNA can also be used for direct RNA sequence analysis.

## 2. Materials

Store all materials at room temperature unless indicated otherwise.

### 2.1. Electrophoresis of RNA Through Formaldehyde-Containing Agarose Gels

1 Low electroendosmosis (EEO) agarose type I (Sigma, St Louis, MO)
2 10X MOPS buffer 0 2*M* 3-*N*-morpholinopropane-sulphonic acid (Sigma), 50 m*M* sodium acetate, 10 m*M* EDTA, adjusted to pH 7 0 with NaOH Autoclave (**Note:** The solution will appear yellow after autoclaving This has no apparent detrimental effect )
3 Formaldehyde, 37% (v/v) solution in $dH_2O$ **Caution:** Formaldehyde solution and vapors are extremely toxic The solution should be stored in a ventilated area or a fume hood but not in the same location as hydrochloric acid It should be used in a fume hood, but not by a person under 18 yr old
4 1% (w/v) Glycine solution
5 Formamide Puriss, assay >99% (obtained from Fluka, Dorset, UK) **Caution:** Formamide is a teratogen and is toxic by inhalation or contact with the skin Take extreme care Formamide dissolves certain types of plastic and can pass through disposable gloves Should not be used by persons under 18 Store at –20°C (*see* **Note 4**)

### 2.2. Glyoxal Treatment of RNA and Electrophoresis Through Tris-Acetate Agarose Gels

1 30% (w/v) Glyoxal solution **Caution:** Glyoxal is an irritant to the skin, eyes, and respiratory system
2. 10X TEAc buffer 0 4*M* Tris base, 0 2*M* sodium acetate, 20 m*M* EDTA Adjust pH to 7 5 with glacial acetic acid Autoclave before use
3 10% (w/v) Sodium dodecyl sulfate (SDS) solution
4. GFP solution 80% (v/v) deionized formamide, 0 75*M* deionized glyoxal in 10 m*M* sodium phosphate, pH 7 0 Make small volumes and dispense into 100-µL aliquots Store at –70°C
5. 5X Loading dye. 20% (w/v) Ficoll 400, 1% (w/v) orange G, 5 m*M* EDTA, pH 7 0 Autoclave Store aliquots frozen at –20°C
6. 0 1*M* NaOH.
7 0 15*M* Sodium acetate (NaOAc), pH 5 5
8 Ethidium bromide solution Stock 10 mg/mL in $dH_2O$ **Caution:** Extreme care Ethidium bromide is a powerful mutagen and is moderately toxic Take appropriate measures to dispose of solid waste correctly (*see* **Note 5**) Store at 4°C

### 2.3. 3' End Labeling of RNA

1 [5'-$^{32}$P] cytidine 3',5' *bis*(phosphate) 2000–4000 Ci/mmol (74–148 TBq/mmol) Store at –20°C. Take standard precautions for handling radioactive material
2 T4 RNA ligase, RNase-free, 3–15 U/μL
3 Dimethyl sulfoxide (DMSO), 99 9%, spectrophotometric grade Handle in fume cupboard
4. 0.5*M* HEPES, adjusted to pH 8 3 with KOH
5 0 1*M* $MgCl_2$
6 0 1*M* Dithiothreitol (DTT) Store at –20°C Labile
7 0 2 m*M* ATP. Store at –20°C
8. Sephadex G-50. Suspend in approx 50 vol TE (10 m*M* Tris-HCl, pH 7 5, 1 m*M* EDTA) Leave overnight Autoclave

### 2.4. Determination of 3'-Terminal Nucleotide

1 1 0*M* NaOH
2. Dye mix containing 0 05% (w/v) each of orange G, acid fuchsin, and xylene cyanol FF in water
3 Whatman 3MM paper
4. Paper electrophoresis buffer, pH 3 5, containing 5% (v/v) glacial acetic acid, 0 5% (v/v) pyridine, and 1 m*M* EDTA **Caution:** All solutions containing pyridine should be handled in a fume cupboard and gloves should be worn at all times
5 X-ray film

## 3. Methods

### 3.1. Electrophoresis of RNA Through Formaldehyde-Containing Agarose Gels

1 Wash gel apparatus, comb, and spacers with detergent, and rinse well Give a final rinse with distilled water Dry with 70% (v/v) ethanol solution
2 Set up apparatus on a level surface in a fume hood (*see* **Note 2**)
3 Melt 0.5 g of agarose in approx 30 mL sterile distilled water Cool to about 55–60°C and add 5 mL 10X MOPS buffer and 3 75 mL 37% (v/v) formaldhyde solution Make up to 50 mL with sterile $dH_2O$
4 Pour gel into mold and leave to set for approx 30 min.
5 Mix RNA sample in ratio 1.3 (v/v) with solution containing 67 parts deionized formamide solution, 20 parts formaldehyde solution, and 13 parts 10X MOPS buffer and 100 μg/mL ethidium bromide Heat to 60°C for 5 min in sealed Eppendorf tube Immediately cool on ice Spin solution to bottom of tube in a microcentrifuge Add 0 2 vol loading dye
6 Remove comb and end-spacers Load whole of sample on gel Run gel submerged in 1X MOPS buffer (Minigels will take approx 45 min when run at a constantb 80 mA.)
7 At the end of the run, wash gel briefly in $dH_2O$ Observe under ultraviolet illumination (*see* **Notes 3** and **6**)

### 3.2. Glyoxal Treatment of RNA and Electrophoresis Through Tris-Acetate Agarose Gels

1 Make 1.4% (w/v) solution of agarose in 1X TEAc containing 0 1% (w/v) SDS Autoclave (Stocks can be prepared in advance and will store well in an unopened container at room temperature )
2. Prepare and set up apparatus as above (No need to position in a fume hood ) Melt agarose in a microwave and pour into mold Leave to set
3 Denature 1 vol RNA sample with 9 vol GFP solution Heat to 55°C for 15 min. Cool Spin briefly
4 Add 0 2 vol loading dye and load onto gel Cover gel with 1X TEAc buffer and run at 3 V/cm for 2 h, or until dye reaches the end of the gel
5 Soak for 15 min in 0.1*M* NaOH (*see* **Note 7**)
6 Stain with 0 15*M* NaOAc, pH 5 5, containing 4 μg/mL ethidium bromide
7 Destain with $dH_2O$ Observe bands under UV illumination (*see* **Note 6**)

### 3.3. 3' End Labeling of RNA

1 Make up 20 μL of a reaction mixture containing 5 μg RNA and 50 μCi (1 85*M*Bq) [5'-$^{32}$P]pCp 50 m*M* HEPES-KOH, 10 m*M* $MgCl_2$, 3 3 m*M* DTT, 20 μ*M* ATP, 10% (v/v) DMSO
2. Start ligation reaction by adding 5 U of T4 RNA ligase Incubate reaction mixture overnight at 4°C
3 Separate labeled RNA from unincorporated [5'-$^{32}$P]pCp by spinning through columns of Sephadex G-50
4 To the first peak of radioactivity which comes off the column (excluded volume), add 0 1 vol 3*M* NaOAc, pH 5 5, 2 5 vol ethanol Precipitate RNA overnight at –20°C
5 Centrifuge to recover RNA, wash the pellet with 70% (v/v) ethanol, and dry in a vacuum desiccator
6 Redissolve dried pellet in 20 μL of sterile $dH_2O$

### 3.4. Determination of 3'-Terminal Nucleotide

1 To 2 μL of pCp-labeled RNA, add 2 μL of 1*M* NaOH and 6 μL of $dH_2O$ Incubate the reaction mixture overnight at 37°C
2 Apply the sample as a 1-cm-wide strip 15 cm from one end of a 58-cm piece of Whatman 3MM paper Spot 1–2 μL of dye mix on either side of the sample
3 Wet the paper with pH 3.5 paper electrophoresis buffer. To ensure the sample does not move during the wetting process, allow the buffer to approach the origin from both sides simultaneously Blot excess buffer off the paper
4 Place paper in suitable electrophoresis tank (*see* **Note 9**) and electrophorese at 3000 V for 90 min The negatively charged mononucleotides migrate toward the anode
5 Remove paper from tank and allow to dry thoroughly in fume hood
6 Mark the position of the dye markers with ink to which a small amount of $^{35}$S has been added. The orange G (orange) and acid fuchsin (pink) dyes run with nearly

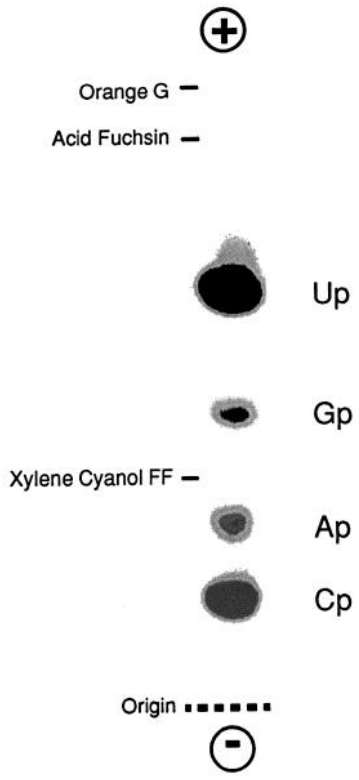



Fig. 1. Separation of $^{32}$P-labeled ribonucleoside monophosphates after electrophoresis on Whatman 3MM paper at pH 3.5. The position of the dye markers is indicated on the left.

the same mobility; the xylene cyanol (blue) migrates about half the distance (**Fig. 1**).

7. Autoradiograph the paper overnight.
8. The nature of the labeled nucleotide can be deduced by comparing its mobility with that of the dye markers (**Fig. 1**).

## 4. Notes

1. The most important rule when working with RNA is that it is essential to take all possible steps to avoid the degradative activity of RNases. If care is not taken, the enzymatic activity can be introduced into an experiment not only from the method of preparation, but also from a number of outside sources. Disposable plasticware (yellow tips, and so on) should be sterilized before use and should not be left exposed to the air. The hands of laboratory workers are a major source of contamination by RNases; investigators should wear disposable gloves at all times

while handling RNA Solutions should be made using autoclaved distilled water, or, whenever possible, autoclaved in suitable glass containers. (**Caution:** Toxic materials or volatile liquids should not be autoclaved )

2 **Caution:** Fumes can be given off formaldehyde gels Ideally they should be run in a fume hood, or at least run with a lid

3 Formaldehyde gels do not stain well if ethidium bromide is added after electrophoresis is completed. If this is done, the bands are normally obscured by a general fogginess over the gel However, this can be partially overcome if the gel is washed with 1% (w/v) glycine solution for 60 min prior to staining

4 Many labs have reported that poor quality formamide is the most likely source of degradation of RNA Our experience has shown that the one listed here is good quality As a matter of routine, however, it is essential that the solution is deionized by stirring for approx 30 min with 40 g/L Amberlite monobed mixed resin (BDH, Poole, Dorset, UK, Amberlite IRN-150L, formerly MB1) The resin is removed by filtration through Whatman no 1 filter paper The efficacy of the resin can be monitored by observation of a drop in pH If there is no fall to neutral pH within this time, it is likely that the resin is exhausted and should be replaced with a fresh batch

5 Gloves should be worn at all times when handling ethidium bromide solutions Also, these solutions should be decontaminated by mixing with activated charcoal after use Filter solid and destroy by incineration The filtrate can be discarded into the drains Gels containing the dye should also be destroyed by incineration

6 Ultraviolet radiation is dangerous to the eyes Ensure that suitable eye protection is worn at all times when working with UV light

7 Glyoxal-modified RNA does not stain with ethidium bromide Thus, it is essential to reverse the modification by soaking the gel in 0 1*M* NaOH before staining

8 The condition of the RNA after the labeling reaction can be monitored by electrophoresing a small portion of it on a formaldehyde-containing gel, as described in **Subheading 3.1.** After examination of the gel under UV light, it can be dried-down and autoradiographed to confirm that the $^{32}P$ label is associated with full-length RNA

9 The design of a suitable paper electrophoresis tank can be found in Brownlee *(5)*

10 The remaining pCp-labeled RNA is suitable for use in a variety of direct RNA sequencing protocols, such as those described by Donis-Keller et al *(6)* and Peattie *(7)*

## References

1 Boedtker, H (1971) Conformation independent molecular weight determinations of RNA by gel electrophoresis *Biochim Biophys Acta* **240,** 448–453

2 Lehrach, H , Diamond, D., Wozney, J. H , and Boedtker, H (1977) RNA Molecular Weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination *Biochemistry* **16,** 4743–4751

3. McMaster, G K. and Carmichael, G G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc Natl Acad Sci USA* **74,** 4835–4838
4. England, T. E and Uhlenbeck, O C (1978) 3'-terminal labelling of RNA with T4 RNA ligase *Nature* **275,** 560–561
5 Brownlee, G G. (1972) *Determination of Sequences in RNA* North Holland, Amsterdam
6 Donis-Keller, H., Maxam, A , and Gilbert, W (1977) Mapping adenines, guanines and pyrimidines in RNA. *Nucleic Acids Res* **4,** 2527–2538
7 Peattie, D A (1979) Direct chemical method for sequencing RNA. *Proc Natl Acad Sci USA* **76,** 1760–1764.

# 26

# RNA Fractionation by Density Gradient Centrifugation

**Michael Shanks**

## 1. Introduction

As shown in previous chapters, many RNA plant viruses have multipartite genomes, which are divided between two or more viral nucleoprotein components. Each nucleoprotein component will display different sedimentation properties, according to the size of RNA. Thus, it is possible to fractionate RNA by density gradient centrifugation. This method is particularly useful, since not only are other possible contaminants removed from the virus preparation, but also the RNA is packaged as a nucleoprotein complex and has less chance of being attacked by degradative enzymes. Two approaches are generally recognized The first, and the one most commonly applied to spherical viruses, is used where the different components have different protein·RNA ratios. Isopycnic ultracentrifugation of virus preparations in caesium chloride (or similar density medium) gradients separates the viral components according to their buoyant density. A sample of the virus is carefully layered on to the top of the gradient and is centrifuged at very high speed. During centrifugation, the virus will move down the gradient until it reaches a level where density of the medium equals the density of the virus. When the run is complete, the separated components can be visualized by shining a beam of light directly through the length of the tube; components can then be removed individually by puncturing the side of the tube with a syringe fitted with a wide bore needle. Once the density gradient medium is removed, the RNA is extracted by protocols applicable to each particular virus.

In the second method (and the one normally applied to rod-shaped viruses), separation of the components is dependent on the conformation and shape of the particle, and can include naked RNA. In this technique, during high-speed

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

centrifugation, the molecules move along the gradient at various rates. Larger molecules (i.e., those that have a larger sedimentation coefficient) will migrate faster down the tube than smaller ones. The density gradient medium, in this case, is usually sucrose (often in the range 10–40%). Fractions of the gradient are collected manually and the virus (or RNA) repelleted by a second round of centrifugation

The protocols outlined below describe our preferred methods when working with CPMV (and other comoviruses). Normally, one cycle of buoyant density centrifugation is sufficient to fractionate the RNAs in a suitable form for cDNA synthesis. However, we also carry out a second round of density gradient centrifugation in those cases when it is essential to have very pure RNA (e.g , for experiments involving the inoculation of protoplasts with viral RNA)

## 2. Materials

### 2.1. Separation of Viral Nucleocomponents by Buoyant Density Centrifugation and Extraction of RNA

1 30, 40, 50, and 60% (w/v) Nycodenz (obtained from Nycomed, Oslo, Norway) solutions buffered in 10m*M* sodium phosphate solution, pH 7.0 Autoclave Store at 4°C (*see* **Note 1**)
2 Beckman Ultra-Clear SW40Ti and Type 40 centrifuge tubes (or similar).
3 10 m*M* sodium phosphate buffer, pH 7 0
4. NET buffer 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 7 4, 1 m*M* EDTA. Autoclave.
5 10% (w/v) sodium dodecyl sulfate (SDS) solution
6 1·1 mixture of phenol chloroform solution. **Caution:** Phenol is extremely toxic and caustic Chloroform is a known carcinogen and volatile Use only in a fume hood Store in dark bottles at 4°C Dispose of all waste correctly

### 2.2. Fractionation of RNA by Velocity Centrifugation on Sucrose Gradients

1 15, 20, 25, and 30% (w/v) sucrose solutions buffered in NET solution (as described in **Section 2.1.**) containing 0 1% (w/v) SDS solution Autoclave with care (*see* **Note 4**)
2. Beckman SW40Ti tubes.
3 3*M* Na acetate, pH 5 5

## 3. Methods

### 3.1. Separation of Viral Nucleoproteins by Bouyant Density Centrifugation and Extraction of RNA

1 Carefully layer 2 75 mL of each Nycodenz solution in a SW40Ti tube (heaviest one first).
2 Allow to diffuse overnight at room temperature

3 Load 0.5–0 75 mL (7.5–10 mg) of virus suspension on top surface of the gradient
4 Centrifuge sample at 163,500*g* for 23 h at 15°C
5 Observe separated viral components with a beam of white light shining directly through the length of the tube Remove bands of interest using syringe fitted with a 21-gage needle
6 Dilute virus suspension by adding at least 9 vol of 10 m*M* sodium phosphate solution. Spin in a Beckman Type 40 tube at 93,000*g* for 4 h at 4°C (*see* **Note 2**)
7 Extract RNA by resuspending the pellet in NET buffer, made 2% (w/v) with respect to SDS Warm to 55°C After 2–3 min, add 1 vol phenol chloroform and vortex vigorously. Separate two phases by low speed centrifugation and remove top (aqueous) layer to a fresh tube (*see* **Note 3**)
8 To precipitate the RNA, add 2.5 vol absolute ethanol to aqueous phase and freeze overnight at –20°C (or 30 min at –70°C).
9 Pellet the RNA by centrifuging the sample at 12,000*g* for 10 min Wash with absolute alcohol, recentrifuge for 5 min and dry briefly in a vacuum desiccator Dissolve RNA in sterile $dH_2O$ and store at –70°C. Examine on denaturing agarose gel (**Fig. 1**)

### 3.2. Fractionation of RNA by Velocity Centrifugation on Sucrose Gradients

1 Layer 2 75 mL of each sucrose solution in a SW40Ti centrifuge tube
2 Allow to diffuse for 3–4 h at room temperature (or at 4°C overnight)
3 Load approx 50 µg RNA on to the top surface of the gradient
4 Spin at 130,000*g* for 12 h at 15°C
5 Puncture the bottom of the tube with a needle and collect approx 0 4 mL fractions in sterilized large microcentrifuge tubes.
6 Determine the presence of the RNA by removing a small sample (5-µL aliqout) of each of the fractions and electrophorese on a formaldehyde (denaturing) agarose gel (*see* Chapter 25)
7 Pool appropriate fractions and precipitate the RNA by adding 0.1 vol 3*M* Na acetate soution, pH 5 5, and 2 5 vol absolute ethanol
8 Pellet RNA in a microcentrifuge and wash with absolute alcohol Respin and dry pellet and dissolve in sterile $dH_2O$. Store at –70°C

## 4. Notes

1 Nycodenz is a nonionic derivative of benzoic acid (systematic name 5-(*N*-2, 3-dihydroxypropylacetamido)-2, 4, 6-tri-iodo-*N*, *N'-bis*(2,3 dihydroxypropyl) isophthalamide The original experiments characterizing the use of Nycodenz for isopycnic centrifugation of plant viruses have previously been described by Gugerli *(1)*. Our experience has shown that this material is the most suitable density gradient medium for separation of comovirus components. Initial experiments in our laboratory showed that CPMV nucleoproteins will separate well in CsCl gadients, but when the RNA is extracted from them, it is often degraded and of very poor quality This has never been the case when Nycodenz is used

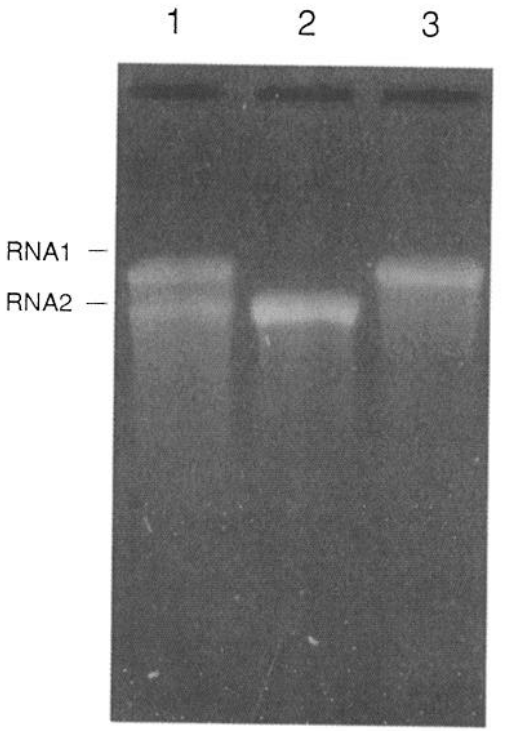



Fig. 1. 1.2% formaldehyde/MOPS agarose gel of CPMV RNAs extracted from whole virus (lane 1) and the two nucleoprotein components separated by ultracentrifugation on a Nycodenz gradient (lanes 2 and 3). Each track contains approx 1 μg of RNA. The position of RNA-1 and RNA-2 is indicated on the left. The RNAs were stained with ethidium bromide and photographed under UV light.

2. Nycodenz absorbs strongly at 260 nm. Hence, it is impossible to determine the concentration of each component by optical density after the first centrifugation. Dilute the suspension and respin as detailed in Methods. If required, separated components can be stored in the same way as whole virus (usually this means the pellet is resuspended in 10 m*M* phosfate buffer and stored at 4°C).
3. Phosphate ions are extremely insoluble in ethanol solutions. Avoid resuspending the separated components in phosphate buffer before RNA extraction.
4. Sugar solutions will brown or caramelize if heated at high temperatures for long periods. Ensure that solutions are autoclaved for no more than 15 min at 121°C.

## Reference

1. Gugerli, P. (1984) Isopycnic centrifugation of plant viruses in Nycodenz density gradients. *J. Virol. Methods* **9**, 249–258.

# 27

# cDNA Library Construction for the Lambda ZAP®-Based Vectors

**Marjory A. Snead, Michelle A. Alting-Mees, and Jay M. Short**

## 1. Introduction

Because the vast majority of plant viruses have a positive-sense RNA genome, which acts as the viral mRNA, the RNA must first be converted into cDNA before cloning, amplification, and subsequent manipulation. Successful cDNA synthesis should yield full-length copies of the original population of mRNA molecules. Hence, the quality of the cDNA library can be only as good as the quality of the mRNA. Pure, undegraded mRNA is essential for the construction of large, representative cDNA libraries *(1)*. Secondary structure of mRNA molecules can cause the synthesis of truncated cDNA fragments. In this case, treatment of the mRNA with a denaturant, such as methylmercuric hydroxide, prior to synthesis may be necessary *(2)*. Other potential difficulties include DNA molecules contaminating the mRNA sample DNA can clone efficiently and their introns can confuse results. RNase-free DNase treatment of the sample is recommended.

After synthesis, the cDNA is inserted into an *Escherichia coli*-based vector (plasmid or λ) and the library is screened for clones of interest. Since 1980, λ has been the vector system of choice for cDNA cloning *(3–10)*. The fundamental reasons are that in vitro packaging of λ generally has a higher efficency than plasmid transformation and λ libraries are easier to handle (amplify, plate, screen, and store) than plasmid libraries. But, most λ vectors have the disadvantage of being poorer templates for DNA sequencing, site specific mutagenesis, and restriction fragment shuffling, although this trend is reversing to some degree with the continued development of PCR techniques.

The development of excisable λ vectors, such as those based on restriction enzyme digestion *(11)*, site-specific recombination *(12)*, or filamentous phage

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

```
5'  GAGAGAGAGAGAGAGAGAGAGACTCGAGTTTTTTTTTTTTTTTTT  3'
      Protective Sequence      XhoI          Poly(dT)
```

Fig 1. 48-bp oligonucleotide hybrid oligo(dT) linker-primer

replication *(13)*, has increased the flexibility of DNA cloning. Now it is possible to clone and screen libraries with the efficiency and ease of λ systems and to analyze positive clones with the ease and versatility of a plasmid The vectors that are compatible with the cDNA synthesis protocol described in this chapter are based on the Lambda ZAP® excision system (Stratagene Cloning Systems, La Jolla, CA) (**refs.** *13* and *14*, manuscript in preparation for SeqZAP) These vectors use an excision mechanism that is based on filamentous helper phage replication (e.g , M13). The choice of vector (Lambda ZAP, ZAP Express, or SeqZAP) depends on whether one requires features such as prokaryotic expression, eukaryotic expression, in vitro transcription, in vitro translation, directional cloning, single-strand replication, automated sequencer compatibility, and special antibiotic resistance selection

Several cloning procedures for constructing cDNA libraries exist *(15–19)* Here we describe a modification of a directional cDNA cloning protocol *(16)*. This procedure has been successfully used for generating hundreds of directional cDNA libraries representing a vast number of plant and animal species containing poly(A)$^+$ mRNA, and is ideal for the generation of cDNA libraries to viral RNA from purified virus particles and RNA extracted from infected plant tissue.

A hybrid oligo(dT) linker-primer containing an *Xho*I site is used to make directional cDNA. This 48-base oligonucleotide was designed with a protective sequence, to prevent the *Xho*I restriction enzyme recognition site from being damaged in subsequent steps, and an 18-base poly(dT) sequence, which binds to the 3' poly(A) region of the mRNA template (*see* **Fig. 1**).

First-strand synthesis is primed with the linker-primer and is transcribed by reverse transcriptase in the presence of nucleotides and buffer. An RNase H-deficient reverse transcriptase may produce larger yields of longer cDNA transcripts *(20,21)*. The use of 5-methyl dCTP in the nucleotide mix during first-strand synthesis "hemimethylates" the cDNA, protecting it from digestion during a subsequent restriction endonuclease reaction used to cleave the internal *Xho*I site in the linker-primer

The cDNA/mRNA hybrid is treated with RNase H in the second-strand synthesis reaction. The mRNA is nicked to produce fragments that serve as primers for DNA polymerase I, synthesizing second strand cDNA. The second strand nucleotide mixture is supplemented with dCTP to dilute the 5-methyl

dCTP, reducing the probability of methylating the second strand, since the *Xho*I restriction site in the linker-primer must be susceptible to restriction enzyme digestion for subsequent ligation into the vector

The uneven termini of the double-stranded cDNA must be polished with cloned *Pfu* DNA polymerase, to allow efficient ligation of adaptors *(22,23)*. Adaptors are complementary oligonucleotides that, when annealed, create a phosphorylated blunt end and a dephosphorylated cohesive end. This double-stranded adaptor will ligate to other blunt termini on the cDNA fragments, and to other adaptors Since the cohesive end is dephosphorylated, ligation to other cohesive ends is prevented. After the adaptor ligation reaction is complete and the ligase has been inactivated, the molecules are phosphorylated to allow ligation to the dephosphorylated vector.

An *Xho*I digestion releases the adaptor and protective sequence on the linker-primer from the 3' end of the cDNA. These fragments are separated from the cDNA on a size-fractionation column. The purified cDNA is then precipitated and ligated to the vector. This strategy is illustrated in **Fig. 2.**

## 2. Materials

### 2.1. First-Strand Synthesis

1 10X first-strand buffer 500 m*M* Tris-HCl, pH 7.6, 700 m*M* KCl, 100 m*M* $MgCl_2$
2 First-strand methyl-nucleotide mixture (10 m*M* dATP, dGTP, dTTP, and 5 m*M* 5-methyl dCTP)
3. Linker-primer (3 0 μg at 1.5 μg/L)
4 Diethylpyrocarbonate (DEPC)-treated water.
5 Ribonuclease inhibitor (40 U)
6. Poly(A)$^+$ mRNA (5 0 μg in ≤36 μL DEPC-treated water, *see* **Notes 1** and **2**)
7 [α-$^{32}$P]-labeled deoxynucleotide (800 Ci/mmol) [α-$^{32}$P]dATP, [α-$^{32}$P]dGTP, or [α-$^{32}$P]dTTP. Do not use [α-$^{32}$P]dCTP (*see* **Note 3**)
8 Reverse transcriptase (250 U) (RNase H-deficient is recommended *[20,21]*)

### 2.2. Second-Strand Synthesis

1 10X second-strand buffer· 700 m*M* Tris-HCl, pH 7 4, 100 m*M* $(NH_4)_2SO_4$, 50 m*M* $MgCl_2$
2 Second-strand dNTP mixture (10 m*M* dATP, dGTP, dTTP, and 26 m*M* dCTP).
3 *E coli* RNase H (4 0 U)
4 *E coli* DNA polymerase I (100 U).

### 2.3. Blunting the cDNA Termini

1 Blunting dNTP mixture (2 5 m*M* dATP, dGTP, dTTP, and dCTP)
2 Cloned *Pfu* DNA polymerase (5 U)
3 Phenol-chloroform (1.1 [v/v], pH 7 0–8 0) (*see* **Note 5**)
4 Chloroform

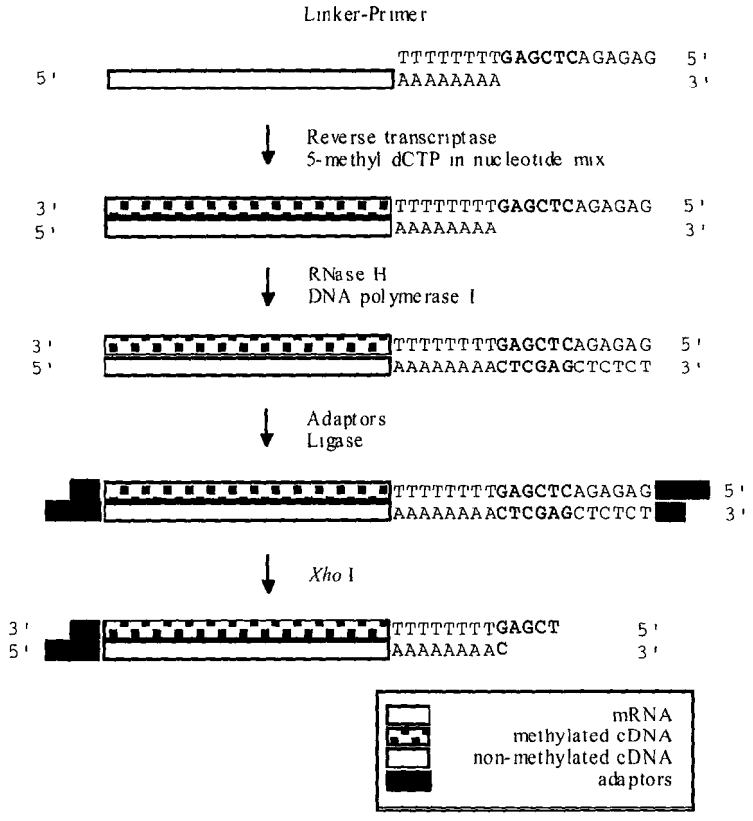



Fig 2 Directional cloning strategy

5. 3*M* Sodium acetate.
6 100% (v/v) Ethanol

## 2.4. Ligating the Adaptors

1 70% (v/v) Ethanol
2 Adaptors (4 μg at 0 4 μg/μL)
3 5% Nondenaturing acrylamide gel
4 10X Ligation buffer. 500 m*M* Tris-HCl, pH 7.4, 100 m*M* $MgCl_2$, 10 m*M* dithiothreitol (DTT)
5. 10 m*M* rATP.
6 T4 DNA ligase (4 Weiss U)

## 2.5. Phosphorylating the Adaptors

1 10X Ligation buffer (*see* **Subheading 2.4., item 4**)
2 10 m*M* rATP
3 T4 polynucleotide kinase (10 U)

### 2.6. XhoI Digestion

1 *Xho*I reaction buffer. 200 m*M* NaCl, 15 m*M* $MgCl_2$
2 *Xho*I restriction endonuclease (120 U)
3 10X STE buffer 1*M* NaCl, 100 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA

### 2.7. Size Fractionation

1 1X STE buffer: 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8 0, 1 m*M* EDTA
2. Sephacryl S-500 column filtration medium (Pharmacia)
3 5% Nondenaturing acrylamide gel
4 Phenol chloroform (1 1 [v/v], pH 7 0–8 0) (*see* **Note 5**).
5 Chloroform.
6 100% (v/v) Ethanol

### 2.8. Quantitating the cDNA

1 70% (v/v) Ethanol
2 TE buffer 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
3 0 8% Agarose
4 Ethidium bromide (10 mg/mL)

### 2.9. Ligating the cDNA to Prepared Vector

1 λ Vector (such as Lambda ZAP, ZAP Express, SeqZAP) double digested and dephosphorylated Vectors are digested with *Xho*I and a second restriction enzyme, which leaves ends compatible with the adaptors
2. 10X Ligation buffer (*see* **Subheading 2.4., item 4**).
3. 10 m*M* rATP
4 T4 DNA ligase (4 Weiss U)

### 2.10. Packaging and Plating

1 NZY medium, plates and top agarose: 5 g NaCl, 2 g $MgSO_4$ $7H_2O$, 5 g yeast extract, 10 g NZ amine (casein hydrolysate) per liter Add 15 g agar for plates or add 0.7% (w/v) agarose for top agarose Adjust the pH to 7.5 with NaOH and sterilize by autoclaving
2 Appropriate *E coli* host strains (such as XL1-Blue MRF' or DH5αMCR) freshly streaked on an LB agar plate containing the appropriate antibiotic (*see* **Note 8**)
3 10 m*M* $MgSO_4$.
4 Packaging extract (such as Gigapack® II λ packaging extract [Stratagene] *[23,24]*)
5 SM buffer: 5 8 g NaCl, 2 0 g $MgSO_4$ $7H_2O$, 50 0 mL 1*M* Tris-HCl, pH 7 5, 5.0 mL 2% (w/v) gelatin per liter. Autoclave
6. Chloroform
7. LB agar plates: 10 g NaCl, 10 g bacto-tryptone, 5 g bacto-yeast extract, 15 g agar per liter. Adjust the pH to 7 5 with NaOH and sterilize by autoclaving
8. Isopropyl-β-D-thio-galactopyranoside (IPTG), 0.5*M* in water and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), 250 mg/mL in dimethylformamide (*see* **Note 10**)

### 2.11. Amplification of the Primary Library

1. Packaged and titered primary library
2. Prepared, appropriate *E. coli* host strains.
3. NZY medium, plates, and top agarose. (*see* **Subheading 2.10., item 1**).
4. SM buffer (*see* **Subheading 2.10., item 5**)
5. Chloroform.
6. Dimethylsulfoxide (DMSO)

## 3. Methods

### 3.1. First-Strand Synthesis

The final volume of the first strand synthesis reaction should be 50 µL. Take this into account when determining the volumes necessary.

1. In an RNase-free microcentrifuge tube, add the reagents in order: 5.0 µL 10X first-strand buffer, 3.0 µL methyl-nucleotide mixture, 2.0 µL linker-primer (1.5 µg/µL), *X* µL DEPC-treated water, 40 U ribonuclease inhibitor.
2. Mix the reagents well. Add *X* µL of poly(A)$^+$ mRNA (5 µg) and gently vortex (*see* **Notes 1** and **2**).
3. Allow the mRNA template and linker-primer to anneal for 10 min at room temperature.
4. Add 0.5 µL of [α-$^{32}$P]-labeled deoxynucleotide (800 Ci/mmol). Do not use [α-$^{32}$P]dCTP (*see* **Note 3**).
5. Add 250 U of reverse transcriptase. The final volume of the reaction should now be 50 µL.
6. Gently mix the sample and briefly spin down the contents in a microcentrifuge.
7. Incubate at 37°C for 1 h.
8. After the 1-h incubation, place on ice.

### 3.2. Second-Strand Synthesis

The final volume of the second strand synthesis reaction should be 200 µL. Take this into account when determining the necessary volumes.

1. To the first-strand reaction (50 µL), add the following components in order: 20.0 µL 10X second-strand buffer, 6.0 µL second-strand dNTP mixture, *X* µL sterile distilled water (DEPC-treated water is not required), 4 U *E. coli* RNase H, 100 U *E. coli* DNA polymerase I.
2. The final volume of the reaction should now be 200 µL. Quickly vortex and spin down the reaction in a microcentrifuge. Incubate for 2.5 h at 16°C.
3. After the 2.5-h incubation, place on ice.

### 3.3. Blunting cDNA Termini

1. Add the following reagents to the synthesized cDNA: 23.0 µL blunting dNTP mixture, 2.0 µL cloned *Pfu* DNA polymerase (2.5 U/µL).

2 Mix well and incubate at 70°C for 30 min Do not exceed 30 min.
3 Phenol.chloroform/chloroform extract (*see* **Note 5**)
4 Precipitate the cDNA by adding the following. 20 µL 3*M* sodium acetate, 400 µL 100% (v/v) ethanol
5 Mix by gently vortexing and incubate on ice for 10 min or overnight at –20°C

## 3.4. Ligating Adaptors

1. Microcentrifuge the precipitated cDNA sample at maximum speed, 4°C for 1 h
2 A large white pellet will form at the bottom of the microcentrifuge tube. Carefully remove the radioactive ethanol and properly discard Counts left in this supernatant are unincorporated nucleotides
3 Wash the pellet by gently adding 500 µL of 70% (v/v) ethanol and microcentrifuge for 2 min
4 Aspirate the ethanol wash and lyophilize the pellet until dry
5. Resuspend the pellet in 9 0 µL of adaptors (0 4 µg/µL) by gentle pipeting. Use a Geiger counter to confirm that the cDNA is in solution
6 Remove 1 0 µL for analysis of cDNA synthesis on a 5% nondenaturing acrylamide gel This aliquot may be frozen at –20°C (*see* **Notes 1–4**)
7. Add the following components to the tube containing the 8.0 µL of blunted DNA and adaptors 1 0 µL 10X ligation buffer, 1 0 µL 10 m*M* rATP, 1 0 µL T4 DNA ligase (4 U/µL)
8 Mix well and briefly spin in a microcentrifuge Incubate overnight at 8°C or for 2 d at 4°C

## 3.5. Phosphorylating the Adaptors

The final volume of the phosphorylation reaction will be 25 µL Take this into account when determining the necessary volumes

1. After ligation, heat inactivate the ligase by incubating at 70°C for 30 min
2. Spin down and allow the reaction to cool at room temperature for 5 min Add 1 5 µL 10X ligation buffer, 2 0 µL 10 m*M* rATP, *X* µL sterile distilled water, 7 U T4 polynucleotide kinase
3 Incubate at 37°C for 30 min.
4. Heat inactivate the kinase by incubating at 70°C for 30 min
5 Spin down and allow the reaction to cool at room temperature for 5 min

## 3.6. *Xho*I Digestion

The final volume of the digestion reaction will be 60 µL.

1 Add the following components to the phosphorylation reaction (25 µL) 30 0 µL *Xho*I reaction buffer, *X* µL sterile distilled water, 120 U *Xho*I restriction endonuclease. Be sure the volume of enzyme is ≤10% of the reaction volume.
2 Incubate for 1 5 h at 37°C
3. Cool the reaction to room temperature and add 15 µL of 10X STE buffer, and 75 µL water

## 3.7. Size Fractionation

There are many types of filtration media used to separate DNA molecules Sephacryl S-500 medium separates efficiently in the 2-kb size range. Drip columns made with Sephacryl S-500 medium separate by size, the larger cDNA molecules eluting from the column first and the small unligated adaptors and unincorporated nucleotides eluting later. The cDNA will not have a high number of counts, but will be detectable by a handheld monitor at ≤250 cps.

### 3.7.1 Drip-Column Preparation

1. Discard the plunger from a 1-mL plastic syringe and insert a small cotton plug Push the cotton to the bottom of the syringe
2. Fill the syringe to the top with Sephacryl S-500 filtration medium
3. Place the syringe in a rack and allow the column to drip "dry"
4. Fill the syringe up to ~0 5 cm from the top with medium, and drip through as in **step 3**
5. Rinse the column with two aliquots of 300 L of 1X STE buffer (total wash volume of 1200 µL). Drip-dry after each addition of buffer

### 3.7.2 Collecting Fractions

1. Pipet the cDNA into the washed Sephacryl S-500 drip column, and allow to drip through This is fraction 1 The recovery volume is ~150 µL and does NOT contain cDNA (*see* **Note 6**)
2. Load two more aliquots of 150 µL of 1X STE buffer on the column and drip through. These are fractions 1 and 3
3. Collect fraction 4 in fresh tube. Load 150 µL of 1X STE buffer and drip as before
4. Collect fraction 5 as in **step 3** Two fractions are usually adequate The size of the cDNA decreases in each additional fraction. Most of the radioactivity will remain in the column owing to unincorporated nucleotides Discard the radioactive drip column appropriately
5. Remove 5 µL from each fraction (or up to 1/10 of the fraction volume) for analysis of cDNA size on a 5% nondenaturing acrylamide gel These aliquots can be frozen at –20°C
6. To remove any residual enzyme from previous reactions, phenol-chloroform/chloroform extract (*see* **Note 5**)
7. Add twice the volume of 100% (v/v) ethanol to precipitate the cDNA
8. Place on ice for 1 h or at –20°C overnight

## 3.8. Quantitating the cDNA

1. Microcentrifuge the fractionated cDNA at maximum speed for 30–60 min at 4°C. Carefully transfer the ethanol to another tube and monitor with a Geiger counter Most of the counts should be present in the pellet Discard the ethanol appropriately

2 Wash the cDNA pellet with 200 µL of 70% (v/v) ethanol and microcentrifuge for 2 min
3 Carefully remove the ethanol wash and vacuum evaporate until the cDNA pellet is dry
4 Each fraction can contain 0–250 cps If the pellet contains 0–10 cps, resuspend the cDNA in 5 0 µL of sterile water If the pellet contains >10 cps, resuspend the cDNA in 12.0 µL of sterile water
5 Quantitate the cDNA by UV visualization of samples spotted on ethidium bromide agarose plates (*see* **Note 7**) The cDNA can be stored at –20°C

## 3.9. Ligating cDNA to Prepared Vector

The cloning vector should be double-digested with *XhoI* and an enzyme which leaves ends compatible with the adaptors. The vector should also be dephosphorylated to prevent vector-to-vector ligations. The final ligation reaction volume is 5 µL.

1 To a 0 5-mL microcentrifuge tube, add in order· *X* µL water, 0 5 µL 10X ligation buffer, 0 5 µL 10 m*M* rATP, 1 µg prepared λ arms, 100 ng cDNA, 0 5 µL T4 DNA ligase (4 Weiss U/µL)
2 Incubate overnight at 4°C

## 3.10. Packaging and Plating

The ligation is packaged and transfected into an appropriate *E coli* host strain

### 3.10.1. Preparation of Plating Cells

1 Inoculate 50 mL of NZY medium with a single colony of the appropriate *E coli* host. Do not add antibiotic
2 Grow at 30°C with gentle shaking (1000*g*) overnight (*see* **Note 9**)
3 Spin the culture at 1000*g* for 10 min.
4 Gently resuspend the cells in 20 mL sterile 10 m*M* $MgSO_4$
5 Determine the concentration of the cells by reading $OD_{600}$ on a spectrophotometer Store this cell stock at 4°C for no more than 1 wk To use, dilute cells to $OD_{600}$ = 1 0 in 10 m*M* $MgSO_4$.

### 3.10.2. Packaging

Package the ligation reaction following manufacturer's instructions. Stop the reaction by adding 500 µL SM buffer and 20 µL chloroform.

### 3.10 3. Plating

1 Mix the following components in a Falcon 2059 polypropylene tube 200 µL appropriate diluted host cells (*see* **Subheading 3.10.1.**), 1 µL final packaged reaction

2 Incubate the phage and the bacteria at 37°C for 15 min to allow the phage to attach to the cells
3 Add 2–3 mL of NZY top agarose (48°C) containing IPTG and X-gal (*see* **Note 10**) Plate onto NZY agar plates and place them upside down in a 37°C incubator
4 Plaques should be visible after 6–8 h Background plaques are blue, recombinant plaques are clear and should be 10- to 100-fold above the background
5 Count the plaques and calculate the titer Primary libraries can be unstable Immediate amplification of at least a portion of the library is recommended to produce a large, stable quantity of a high-titer stock of the library.

### 3.11. Amplification of the Primary Library

After amplification, the library is suitable for screening by a variety of techniques *(2)*. More than one round of amplification is not recommended, since slower growing clones may be significantly underrepresented

1 Prepare the host strains (*see* **Subheading 3.10.1.**)
2 Mix aliquots of the packaged library containing ~50,000 plaque-forming units (PFU) (≤300 µL vol) with 600 µL of host cells in Falcon 2059 polypropylene tubes Usually, $1 \times 10^6$ PFU are amplified (20 tubes)
3 Incubate the tubes containing the phage and host cells for 15 min at 37°C
4 Mix 8 0 mL of melted NZY top agarose (48°C) with each aliquot of infected bacteria and spread evenly onto a freshly poured 150-mm NZY plate
5 Incubate the plates at 37°C for 6–8 h Do not allow the plaques to grow larger than 1–2 mm
6 Overlay the plates with 8–10 mL of SM buffer Store the plates at 4°C overnight with gentle rocking The phage will diffuse into the SM buffer
7 Recover the SM buffer containing the bacteriophage from each plate and pool it in a sterile polypropylene container Add chloroform to a 5% final concentration and mix well
8. Incubate for 15 min at room temperature
9 Remove the cell debris by centrifugation for 10 min at 500*g*
10 Recover the supernatant and transfer it to a sterile polypropylene container Add chloroform to a 0.3% final concentration and store at 4°C.
11 Check the titer of the amplified library by making serial dilutions in SM buffer and plating on host cells (*see* **Subheading 3.10.3.**) The average titer is usually $10^9$–$10^{12}$ PFU/mL
12. Frozen stocks can be made by adding dimethylsulfoxide (DMSO) to a final concentration of 7%, mixing well, and freezing at –80°C

## 4. Notes

1 The mRNA sample must be highly purified for efficient cDNA synthesis The mRNA sample may contain inhibitors that can be removed by phenol·chloroform extractions The presence of DNA or rRNA will give an inaccurate concentration of mRNA, leading to an insufficient amount of sample used Treat the mRNA

with RNase-free DNase or use more mRNA sample

2 Some populations of mRNA molecules may have tight secondary structures Methylmercuric hydroxide treatment of the RNA sample may be necessary Perform the following protocol under a fume hood Resuspend the mRNA in 20 µL of DEPC-treated water and incubate at 65°C for 5 min Cool to room temperature and add 2 µL of 100 m*M* methyl-mercuric hydroxide Incubate at room temperature for 1 min, add 4 µL of 700 m*M* β-mercaptoethanol (dilute stock in DEPC-treated water), and incubate at room temperature for 5 min. The final volume is 26 µL This denatured mRNA is ready for first strand synthesis.

3. Do not use [α-$^{32}$P]dCTP The 5-methyl dCTP present in the nucleotide mixture will be diluted and the synthesized cDNA will not be protected from the subsequent restriction digest Gel analysis may show a false negative result if the [α-$^{32}$P]dNTP is degraded, because it may not incorporate into the cDNA even though synthesis is occurring

4 Gel analysis may show hairpinning of the cDNA, which is caused by a number of factors an insufficient amount of mRNA was used in the first strand reaction (*see* **Note 1**), the mRNA population had tight secondary structure (*see* **Note 2**), the second-strand incubation temperature was higher than 16°C (cool the first strand reaction by placing it on ice before adding the second strand synthesis reaction components), or an excessive amount of DNA polymerase was used in the second strand reaction

5 Phenol·chloroform (1:1 [v/v], pH 7 0–8 0) is recommended Do not use low-pH phenol routinely used for RNA isolation ***(1,2)*** To extract the cDNA sample, add an equal volume of phenol chloroform (1 1 [v/v], pH 7 0–8 0) and vortex Microcentrifuge at maximum speed for 2 min Transfer the upper aqueous layer, which contains the cDNA, to a new sterile tube Avoid removing any interface Add an equal volume of chloroform, and vortex Microcentrifuge for 2 min at maximum speed Save the upper aqueous layer and transfer it to a new tube

6. Sephacryl S-500 drip columns can be run "dry" A reservoir at the top of the column is not required Each 150-µL wash yields an ~150-µL fraction volume Fractions 1–3 can be collected in one tube since these fractions do not contain cDNA. The cDNA elutes in fractions 4 (containing fragments ≥1 5) and 5 (containing fragments >500 bp)

7 Ethidium bromide agarose plate quantitation is performed as follows Using a DNA sample of known concentration (such as a plasmid), make serial dilutions (200, 150, 100, 75, 50, 25, and 10 ng/µL) in TE buffer Melt 10 mL of 0 8% (w/v) agarose in TE buffer and cool to 50°C. Under a hood, add 10 µL of 10 mg/mL ethidium bromide, swirl to mix, and pour into a 100-mm Petri dish Allow the plate to harden Label the bottom of the Petri dish with a marker to indicate where the sample and standards will be spotted Carefully spot 0 5 µL of each standard onto the surface of the plate Do not puncture the agarose Allow capillary action to pull the small volume from the pipet tip to the surface Spot 0 5 µL of the cDNA sample onto the plate adjacent to the standards. Allow the spots to absorb into the agarose for 10–15 min at room temperature. Invert the plate and visual-

ize on a UV lightbox Compare the spotted sample of unknown concentration with the standards to determine the concentration of the cDNA

8 Since the cDNA is heavily methylated, introduction into a host with an McrA, McrCB, hsdSMR, Mrr phenotype would be subject to digestion by these restriction systems. Therefore, the choice of packaging extract and an *E coli* host strain is crucial *(24–29)*

9 Since λ phage can adhere to dead as well as to viable cells, the lower temperature prevents the bacteria from overgrowing.

10 Most cDNA vectors have color selection by Isopropyl-β-D-thio-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) These components can be added to the top agarose before plating to produce the background blue color Use 15 µL of 0 5*M* IPTG (in water) and 50 µL of X-gal at 250 mg/mL in dimethylformamide.

## References

1 Chomczynski, P and Sacchi, N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction *Anal Biochem* **162,** 156–159

2 Sambrook, J , Fritsch, E F , and Maniatis, T , eds (1989) *Molecular Cloning A Laboratory Manual,* 2nd ed Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

3 Han, J H and Ruttei, W J (1987) Lambda gt22, an improved lambda vector for the directional cloning of full-length cDNA *Nucleic Acids Res* **15,** 6304

4 Huynh, T V , Young, R A , and Davis, R W (1985) *DNA Cloning*, vol I (Glover, D. A , ed ), IRL, Oxford, Washington, D C , pp 49–78

5 Meissner, P S , Sisk, W P , and Berman, M. L (1987) Bacteriophage lambda cloning system for the construction of directional cDNA libraries *Proc Natl Acad Sci USA* **84,** 438–447

6 Murphy, A J M and Efstratiadis, A (1987) Cloning vectors for expression of cDNA libraries in mammalian cells *Proc Natl Acad Sci USA* **84,** 8277–8281

7 Palazzolo, M J. and Meyerowitz, E M (1987) A family of lambda phage cDNA cloning vectors, lambda SWAJ, allowing the amplification of RNA sequences *Gene* **52,** 197–206

8 Scherer, G , Telford, J , Baldari, C , and Pirrotta, V (1981) Isolation of cloned genes differentially expressed at early and late stages of Drosophila embryonic development *Dev Biol* **86,** 438–447

9 Young, R A and Davis, R W (1983) Efficient isolation of genes by using antibody probes. *Proc Natl Acad Sci USA* **80,** 1194–1198

10. Young, R A and Davis, R W (1983) Yeast RNA polymerase II genes isolation with antibody probes *Science* **222,** 778–782

11 Swaroop, A. and Weissman, S M (1988) Charon BS (+) and (–), versatile lambda phage vectors for constructing directional cDNA libraries and their efficient transfer to plasmids *Nucleic Acids Res* **16,** 8739

12 Palazzolo, M F , Hamilton, B. A , Ding, D L , Martin, C H , Mead, D H , Mierendorf, R C , Raghavan, K V , Meyerowitz, E M , and Lipshitz, H D (1990)

Phage lambda cDNA cloning vectors for subtractive hybridization, fusion-protein synthesis and Cre-loxP automatic plasmid subcloning *Gene* **88,** 25–36

13 Short, J. M , Fernandez, J M , Sorge, J A , and Huse, W D (1988) Lambda ZAP a bacteriophage lambda expression vector with in vivo excision properties *Nucleic Acids Res* **16,** 7583–7600

14 Alting-Mees, M., Hoener, P , Ardourel, D , Sorge, J A., and Short, J M (1992) New lambda and phagemid vectors for prokaryotic and eukaryotic expression. *Strategies Mol Biol* **5(3),** 58–61.

15 Gubler, U. and Hoffman, B. J (1983) A simple and very efficient method for generating cDNA libraries *Gene* **25,** 263–269

16 Huse, W D. and Hansen, C (1988) cDNA cloning redefined a rapid, efficient, directional method *Strategies Mol Biol* **1(1),** 1–3

17 Kimmel, A R and Berger, S L (1989) Preparation of cDNA and the generation of cDNA libraries. overview *Methods Enzymol* **152,** 307–316

18 Krug, M S and Berger, S L (1989) First strand cDNA synthesis primed with oligo (dT) *Methods Enzymol* **152,** 316–325

19. Okayama, H and Berg, P (1982) High-efficiency cloning of full-length cDNA *Mol Cell Biol* **2,** 161–170

20 Gerard, G (1989) cDNA synthesis by cloned moloney murine leukemia virus reverse transcriptase lacking RNaseH activity *Focus* **11,** 66

21 Nielson, K , Simcox, T G , Schoettlin, W , Buchner, R , Scott, B , and Mathur, E (1993) Stratascript RNaseH-reverse transcriptase for larger yields of full-length cDNA transcripts *Strategies Mol Biol* **6(2),** 45

22 Costa, L , Grafsky, A , and Weiner, M P (1994) Cloning and analysis of PCR-generated DNA fragments *PCR Methods Applications* **3,** 338–345

23 Hu, G (1993) DNA polymerase-catalyzed addition of nontemplated extra nucleotides to the 3' end of a DNA fragment *DNA Cell Biol* **12,** 763–770

24 Kretz, P L , Reid, C H , Greener, A , and Short, J M (1989) Effect of lambda packaging extract mcr restriction activity on DNA cloning *Nucleic Acids Res* **17,** 5409

25 Kretz, P L and Short, J. M (1989) Gigapack™II. Restriction free (hsd-, mcrA-, mcrB-, mrr-) lambda packaging extracts *Strategies Mol Biol* **2(2),** 25,26

26. Bullock, W , Fernandez, J M , and Short, J M (1987) XL1-Blue a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5,** 376–379

27. Kohler, S W , Provost, G S , Kretz, P L , Dycaico, M J , Sorge, J A , and Short, J M (1990) Development of short-term in vivo mutagenesis assay. The effects of methylation on the recovery of a lambda phage shuttle vector from transgenic mice *Nucleic Acids Res* **18,** 3007–3013

28. Kretz, P L , Kohler, S W , and Short, J M (1991) Gigapack® III high efficiency lambda packaging extract with single-tube convenience *Strategies Mol Biol* **7(2),** 44,45.

29. Kretz, P L , Kohler, S W , and Short, J M. (1991) Identification and characterization of a gene responsible for inhibiting propagation of methylated DNA sequences in mcrA and mcrB *Escherichia coli* strains. *J Bacteriol* **173,** 4707–4716

# 28

# PCR Cloning of Coat Protein Genes

**Rebecca Stratford**

## 1. Introduction

The polymerase chain reaction (PCR) is used to amplify DNA between two oligonucleotide primers, of which one is complimentary to a sequence on the (+)-strand and the other to a downstream sequence on the (–)-strand. Amplification between the two primers is achieved by reiterative cycles of template denaturation, primer annealing, and primer extension by a heat-stable DNA polymerase enzyme, which is able to withstand the repeated high temperatures required for DNA denaturation The products of each reaction cycle serve as templates for subsequent cycles, and so, theoretically, the amount of product doubles after each cycle. PCR is therefore a very sensitive technique that can be used to generate microgram quantities of DNA from as little as a single DNA molecule (*see also* Chapter 48).

PCR reactions can also be used to amplify products from RNA templates (RT-PCR; *see also* Chapter 48) The RNA is reverse-transcribed to form a single complementary strand of DNA, which is then amplified by PCR, using primers on either side of the target gene. RT-PCR, using either purified viral RNA or total RNA from infected plants, can be used for cloning genes or for diagnostic purposes *(1,2)*. RT-PCR requires sequence information from both sides of the target gene, but a modification of the method can be used to clone genes from which only limited sequence data is available The RACE-PCR technique developed by Frohman et al. *(3)* enables amplification using a single gene-specific primer combined with a generic primer. First-strand cDNA is synthesized using an oligo dT-adaptor primer, which anneals to the poly A tail present at the 3' end of all eukaryotic mRNAs. The subsequent PCR reaction uses the single gene-specific primer and a second primer corresponding to the adaptor sequence of the cDNA primer (**Fig. 1B**). Consequently, it is possible to clone

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

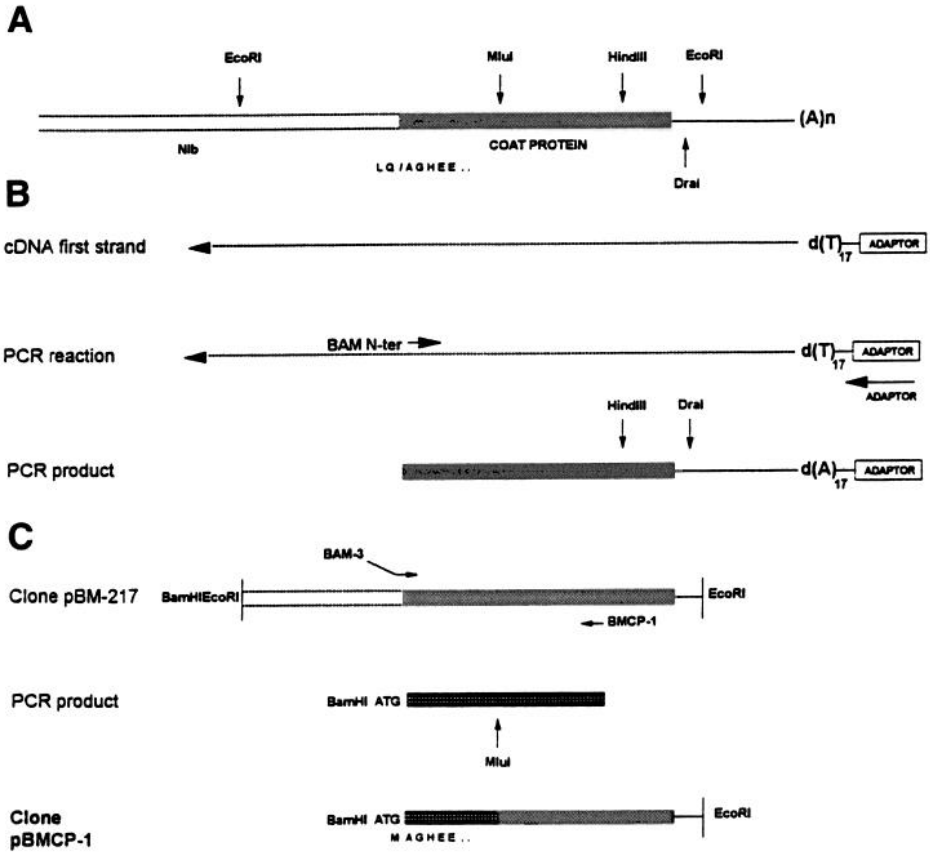



Fig. 1. Diagram of **(A)** 3' end of BaMMV RNA-1 showing the location of the CP gene and the putative polyprotein cleavage site; **(B)** use of RACE-PCR technique to amplify the CP gene using a degenerate primer (BAM N-ter), corresponding to the N-terminal amino acids; **(C)** addition of an ATG start codon to the CP gene present within clone pBM-217.

plant virus coat protein (CP) genes on the basis of N-terminal amino acid sequence data alone.

PCR can also be used to add extra nucleotides to or to change the sequence of a particular gene simply by incorporating the required sequence into the synthetic primer. Nucleotides that are not complementary to the target sequence may be added to the 5' end of a PCR primer without deleterious effect, since the specificity of the reaction is largely determined by annealing of the 3' end to the template DNA. This adaptation has a large variety of appli-

cations; for example, it may be used to add translational start or stop codons, to change the translational context of an existing ATG codon, or simply to add restriction enzyme sites, to facilitate subsequent cloning steps.

## 2. Materials

### 2.1. Reaction Components

PCR amplification requires DNA primers, dNTPs, buffer, $MgCl_2$, *Taq* polymerase, and template DNA. The optimal concentrations of these components, particularly $MgCl_2$, are likely to vary between applications, and often have to be determined experimentally. The range of concentrations most frequently used, and general guidelines, are provided below.

1. Primers PCR primers are most stable when stored as a lyophilized pellet, but for convenience can be stored as a high-concentration stock solution in sterile distilled water (SDW) at –20°C It is best to avoid use of buffers containing EDTA, since *Taq* polymerase requires $Mg^{2+}$ ions, which will be chelated by EDTA Primers are generally used at concentrations between 0 2 and 1 0 μ*M* for each primer High primer concentrations may promote mix-priming and primer-dimer artifacts produced by the interaction of primers alone
2. dNTPs A concentrated stock of dNTPs, with each dNTP at 1.25 m*M*, can be stored in aliquots at –20°C to avoid repeated freeze–thaw cycles PCR reactions generally contain 20–200 μ*M* dNTPs and it is important that all four nucleotides are present at the same concentration to ensure the highest level of fidelity. The specificity and fidelity of PCR is increased by using low dNTP concentrations
3. *Taq* polymerase The recommended range of *Taq* polymerase is 1–4 U/100 μL reaction, but requirements will vary with different templates or primers, and a range of concentrations can be tested. If the concentration is too high, a nonspecific background smear can develop, too little produces low yields A wide range of heat-stable DNA polymerases are now available, including some that have a 3' to 5' exonuclease proofreading activity Also available is a thermostable reverse transcriptase, which can be used to make both cDNA and DNA amplification products in RT-PCR reactions
4. PCR buffer Buffers for PCR reactions, are generally based on 10–50 m*M* Tris-HCl (pH 8.3–8 8) and contain up to 50 m*M* KCl to facilitate primer annealing Gelatin, BSA, or Tween-20 are often added to help stabilize the enzyme, and cosolvents such as DMSO are sometimes also included to facilitate template denaturation PCR buffers are usually supplied with the polymerase and vary in composition, depending on the supplier If a reaction is not working, it is often worth trying either an alternative buffer system or source of polymerase
5. $MgCl_2$ *Taq* polymerase is a magnesium-dependent enzyme, and free $Mg^{2+}$ concentration also can affect primer annealing and strand desiccation Generally, reactions contain 1.5 m*M* $MgCl_2$, but it can be beneficial to optimize the magnesium ion concentration, usually within a 0 5- to 4-m*M* range

### 2.2. Primer Design

It is important to check that the primers are complimentary to the correct template strand and in the appropriate 5' to 3' orientation to produce the desired PCR fragment. In general, PCR primers are designed to be between 20 and 30 bases in length, and to have a 50–60% GC content Where possible, the 3' end of the primer should ideally be a G or C residue to anchor the primer base to the template for efficient elongation, but runs of several Gs and/or Cs should be avoided, since they can promote mis-priming. The primer sequences should be checked for self-complementarily and primer–primer complementarity, since this will significantly affect the efficiency of amplification by competing with the template.

Degenerate primers can be synthesized containing a mixture of more than one base at any position, and are used when amino acid sequence data is the basis for primer design. Amino acids with the minimum degeneracy should be selected, and it helps to avoid degeneracy at the 3' end Inosine, which hybridizes equally well to any of the four bases, can also be used, and this reduces the complexity of the final primer mixture. Computer software programs are available to assist primer design and can be used to check for secondary structure and degree of degeneracy

### 2.3. Minimizing PCR Contamination

The ability of PCR to produce large amounts of DNA from low quantities of template necessitates that extreme care be taken to avoid false positives caused by contamination, for this reason, a negative control reaction should be included in all experiments. The most likely sources of contamination are the carryover of products from a previous PCR reaction or from one sample to the next A number of steps can be taken to reduce these problems. Carryover of products from one reaction to another can be avoided by physically separating the operations performed to set up the reaction from those required to handle the products, by using, for example, a separate pipet dedicated to handling PCR products Aerosol-resistant tips or positive-displacement pipets can be used to prevent contamination caused by aerosols reaching the shaft of conventional pipets. Adding the template DNA last can also help to reduce opportunities for transfer between samples. In addition, aliquoting reagents will limit any contamination to a single experiment.

## 3. Methods

Two methods are described: the use of RACE-PCR to amplify CP genes using only N-terminal amino acid sequence data (*see* **Subheading 3.1.**), and the addition of an ATG start codon to the 5' end of a CP gene (*see* **Subheading**

3.2.). Both methods are illustrated using barley mild mosaic virus (BaMMV), but can be adapted for other viruses by using appropriate primers and template Although the reaction components and conditions provided will amplify many different targets, they are primarily intended as a starting position, and in some cases it will be beneficial to optimize the PCR using the guidelines provided

### 3.1. PCR Amplification of BaMMV CP Gene Using a Single Primer

Since the BaMMV CP is located at the 3' end of RNA-1, which is polyadenylated (**Fig. 1A**), the gene can be cloned using the RACE-PCR technique *(3)*. Purified viral RNA or total RNA from infected plants can be reverse transcribed using a $dT_{17}$ adaptor primer, which anneals to the 3' poly(A) tail. This single-stranded cDNA is then PCR-amplified using the adaptor primer and a single gene-specific primer corresponding to the N-terminus of the CP gene (**Fig. 1B**; **Note 1**).

1 Add 10 µL of purified viral RNA (~1–10 ng/µL) to 100 pmol of the $dT_{17}$-adaptor primer (1 8 µL of 627 µg/mL stock, **Note 2**) and incubate at 70°C for 10 min The tube should be immediately cooled on ice

$dT_{17}$-adaptor primer 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'

The adaptor sequence contains restriction sites that can be used for subsequent cloning of the RACE PCR product Three different sites (*Xho*I, *Sal*I, *Cla*I) are included, so that there is likely to be at least one that does not cut within the product itself, if this is not the case, different sites can be used by resynthesizing an alternative primer Total RNA (~1 µg) from infected plants could also be used as a template

2 A first-strand cDNA reaction is then performed by addition of 5 µL 5X RT buffer (5X stock is 250 m*M* Tris-HCl, pH 8 3, at room temperature, 375 m*M* KCl, 15 m*M* $MgCl_2$, as supplied by Gibco-BRL), 2 5 µL 100 m*M* DTT, 2 5 µL 10 m*M* dNTPs, 0 5 µL RNasin ribonuclease inhibitor (40 U/µL, Promega, Southampton, UK) 1 7 µL SDW; the contents are mixed and incubated at 37°C for 5 min and then add 1 µL of M-MLV RNase H-reverse· transcriptase (200 U/µL, Gibco-BRL). The tube is further incubated at 37°C for 60 min, followed by 42°C for 30 min, and the reaction is stopped by heating to 94°C for 5 min For convenience, the reaction is carried out in a DNA thermocycler, but can be performed using a heated water bath. Control reactions are also performed using either no template (SDW instead of RNA) or without the addition of reverse transcriptase The latter control is to ensure that the PCR products are derived from an RNA template, rather than from contaminating DNA, and is particularly important if total RNA from infected plants is being used, rather than purified viral RNA

3 A 25-µL PCR reaction is performed using an adaptor primer corresponding to the 5' end of the primer used for cDNA synthesis and a degenerate primer

(BAM N-ter) designed from the amino acid sequence of the N-terminus of the BaMMV CP *(4)*.

Adaptor primer 5'-GACTCGAGTCGACATCGA-3'

| | H | E | E | P | D | (P) | |
|---|---|---|---|---|---|---|---|
| BAM N-ter | 5'-CAC | GAA | GAA | CCA | GAC | CC | -3' |
| | T | G | G | C | T | | |
| | | | | G | | | |
| | | | | T | | | |

BAM N-ter contains mixed bases at five positions, to allow for variation in codon usage The adaptor and BAM N-ter primers are estimated to have $T_m$s of 56°C and 55°C, respectively (**Note 3**) The reaction contains 2 5 µL of 10X PCR buffer (10X stock is 200 m*M* $(NH_4)_2SO_4$, 750 m*M* Tris-HCl, pH 9 0, at 25°C, 0 1% [w/v] Tween, supplied by Advanced Biotechnologies, Surrey, UK), 4 µL of 1 25 m*M* dNTPs, 2 5 µL of 25 µg/mL BAM N-ter, 2 5 µL of 25 µg/mL adaptor primer, 1 5 µL of 25 m*M* $MgCl_2$, 0 2 µL of *Taq* polymerase (5 U/µL, Advanced Biotechnologies), 6 8 µL SDW, and 1 µL of first-strand cDNA reaction (**Note 4**) A negative control reaction is also set up using SDW, instead of the cDNA The reactions are set up in 0 5 mL tubes and gently overlaid with 25–50 µL of paraffin oil (Fluka) to prevent evaporation during thermocyling Forty cycles of PCR amplification are carried out (94°C for 30 s, 55°C for 1 min, 72°C for 2 5 min), followed by a 10-min extension at 72°C (**Note 5**) in a thermocycler (OmniGene, Hybaid, Middlesex, UK)

4 5 µL of each PCR reaction are electrophoresed on a 1 3% TAE agarose gel (40 m*M* Tris acetate, 1 m*M* EDTA, pH 8 0) containing 0 5 µg/mL ethidium bromide in both the gel and the electrophoresis buffer, the products are visualized under UV light An ~1100-bp product, corresponding to the expected size for the BaMMV CP gene, plus 3' untranslated region, is produced in the reaction containing viral cDNA (**Fig. 2A**, lane 1), but not in any of the negative-control reactions (**Fig. 2A**, lanes 2–4) To verify that this PCR product contains the BaMMV CP gene, 5-µL aliquots are digested with either *Hin*dIII or *Dra*I, which produced fragments of the expected size (**Fig. 2B**, lanes 1 and 2)

The method described can also be adapted to clone CP genes for which two specific primers are available, by substituting the second specific primer for the adaptor primer in the PCR reaction. The cDNA can be synthesized using either the $dT_{17}$ adaptor or a shorter oligo(dT) primer.

### 3.2. Addition of Methionine Start Codon to BaMMV CP Gene

The BaMMV CP gene is expressed via proteolytic cleavage of a larger precursor, and therefore an ATG start codon must be added to the 5' end of the CP gene for expression in transgenic plants. This was achieved using primer BAM-3,

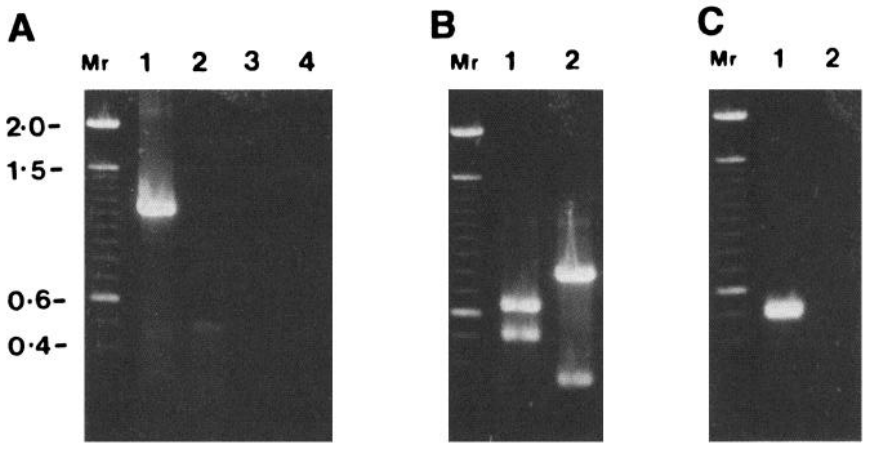



Fig. 2. Amplification products from **(A)** RACE-PCR reaction using first-strand cDNA from purified viral RNA (lane 1) or water as a negative control (lane 4). Further negative-control PCR reactions are shown using the products of a mock first-strand cDNA reaction on viral RNA without reverse transcriptase (lane 2) or without RNA (lane 3). **(B)** Shows the amplification product from (A, lane 1) digested with either *Hin*dIII (lane 1) or *Dra*I (lane 2). **(C)** shows the amplification product of a PCR reaction on clone pBM-217 using primers BAM-3 and BMCP-1. DNA size markers ($M_r$) are shown (100 bp DNA ladder, Gibco- BRL).

which contains an ATG start codon linked to the sequence of the first 18 nucleotides of the CP gene ***(4)***.

BAM-3: 5'-CGC GGATCC AACA ATG GCA GGG CAT GAG GAA CCA -3'
M A G H E E P

The ATG codon is in a favorable plant translation context ***(5)*** and a *Bam*HI restriction site (underlined) was included to facilitate subsequent cloning steps. Since restriction enzymes do not cleave sites close to the end of DNA strands very efficiently, the *Bam*HI site is preceded by a CGC triplet (**Note 6**).

1. A 25-µL PCR reaction is performed on clone pBM-217, which contains the BaMMV CP gene as an *Eco*RI fragment in pUC 13 (**Fig. 1C**). The 5' end of the CP gene is amplified using primer BAM-3 and a second primer BMCP-1 complementary to a region within the gene (*see* **Fig. 1A**).

   BMCP-1: 5'-GGAATAACAGCGGAAGA-3'

   The reaction contains 2.5 µL of 10X PCR buffer (*see above*), 1.5 µL of 25 m*M* $MgCl_2$, 4 µL of 1.25 m*M* dNTPs, 0.2 µL of 5 U/µL *Taq* polymerase (Advanced Biotechnologies), 2.5 µL of each primer (25 µg/mL stock), 1 µL of template DNA

(0 1 ng/μL) and 10 8 μL of SDW (*see* **Note 7**) A second reaction is set up in parallel, with SDW instead of DNA, as a negative control to check for PCR contamination The reactions are set up in 0 5-mL tubes and gently overlaid with 25–50 μL of paraffin oil (Fluka) to prevent evaporation during thermocyling.

2 The reaction tubes are placed in a DNA thermocycler (OmniGene, Hybaid) programmed for 25 cycles of amplification, using a step program (94°C for 30 s, 55°C for 1 min, 72°C for 2 min), followed by a 10-min final extension at 72°C (**Note 5**)

3 10-μL Aliquots of the reaction are loaded onto a 1.3% TAE agarose gel containing 0 5 μg/mL ethidium bromide and products visualized under UV light Amplification of template DNA produces a ~500-bp product corresponding to the expected size (**Fig. 2C**, lane 1), and no products are visible in the negative-control reaction (**Fig. 2C**, lane 2)

4 10 μL of the PCR reaction is digested at 37°C for 2 h with 0 5 μL *Bam*HI and 0 5 μL *Mlu*I (each at 10 U/μL, Gibco-BRL), 2 μL 10X react 3 (Gibco-BRL), and 7 μL of SDW The 280-nt product can be gel purified using the Promega Wizard DNA purification system and an aliquot (~100 ng) ligated overnight at 14°C with ~50 ng of the 3 6 Kb *Bam*HI-*Mlu*I fragment of pBM217 in 1X ligation buffer (50 m*M* Tris-HCl, pH 7 6, 10 m*M* $MgCl_2$, 1 m*M* ATP, 1 m*M* DTT, 5% polyethylene glycol 8000, Gibco-BRL) with 0 5 U T4 DNA ligase (Gibco-BRL) The ligation (0 5 μL) is transformed into *E coli* strain DH5α (Stratagene) following the manufacturer's instructions This cloning step replaces the original 5' 270 nt of the coat protein in pBM217 with the 5' end of the PCR product to produce a clone containing the BaMMV CP gene with an ATG start codon in a good translational context (pBMCP-1, **Fig. 1C**) The 5' end of the clone should be sequenced to verify the presence of the ATG start codon and to ensure that no nucleotide changes had been introduced into the CP fragment by misincorporation during the PCR amplification (*see* **Note 8**).

## 4. Notes

1 The quality of the RNA preparation is vital to the success of this technique, and care must be taken not to contaminate the sample with RNase during extraction and subsequent handling All tubes and solutions should be autoclaved, and gloves should be worn. The most likely problems are lack of product or generation of false products because of nonspecific amplification Mismatched annealing may be reduced by increasing the anneal temperature or decreasing the primer concentration. The lack of a product may be caused by RNase contamination or the presence of secondary structure in the RNA template, causing premature termination of the reverse transcription reaction This can be overcome by using a higher temperature, e g , 52°C, for the reaction, the temperature can be increased further if a thermostable reverse transcriptase is used Although RACE-PCR is most frequently used to amplify genes with poly(A) tails, CP genes from nonpolyadenylated viruses could be amplified by using terminal transferase to add a poly(A) tail to the genomic RNA.

2 The concentration of primers is estimated by measuring the absorbance of an aliquot at 260 nm, since a 33-μg/mL solution of ssDNA equals 1 $A_{260}$ unit For example, 10 μL of the adaptor primer was diluted with 990 μL of SDW and the $A_{260}$ was 0.19 U Since the sample was diluted 100×, the concentration of the stock solution is 0.19 × 100 × 33 μg/mL i e , 627 μg/mL Often, it is necessary to calculate the molarity of primers, a rule of thumb is· 1 μg of primer = 1 52 × (2000/*n*) pmol, where *n* = the number of bases For example, the $dT_{17}$ adaptor primer is 35 bases long and is at 627 μg/mL Therefore, 1 μg of primer contains approx 87 pmol, 1 15 μg of primer contains 100 pmol, and so 1 8 μL of the 0.627 μg/μL stock is required for the cDNA reaction

3. The $T_m$ of a primer is the temperature at which 50% of the primer is annealed to its complementary target sequence; this figure is used as a starting point in determining the optimum annealing temperature. A rule of thumb for calculating the $T_m$ of a primer is. $T_m$ = (4 × number of G + C residues) + (2 × number of A + T residues) For example, the adaptor primer contains 10 G + C bases and 8 A + T bases, and so the $T_m$ is approx 56°C, i e., the sum of (4 × 10) + (2 × 8) It is desirable to construct primers with roughly equal $T_m$s, but in practice this is not always possible The annealing temperature for a PCR reaction is usually 2–5°C below the $T_m$, but can be increased above the $T_m$, to increase the specifity of the reaction if required At normal primer concentrations (0 2–1 0 μm), the annealing step requires only a few seconds.

4 In practice, it is more convenient and accurate to make up a cocktail containing all reaction components except the template DNA The cocktail is then aliquoted and the template added separately to each tube to minimize the opportunity for crosscontamination from sample to sample

5. A wide range of DNA thermocyclers are now available, and the best machines have good uniformity of temperature over the block, reproducible heating, and cooling rates, and are set up to ensure that the contents of the tube (rather than just the block itself) reach the programmed temperature Specially designed thin-wall PCR tubes can also be used for fast heat transfer Most PCR reactions are performed as step programs in which the machine moves from one temperature to the next at the maximum rate, but with many machines it also possible to control the rate of heating and cooling, as required The temperatures and length of time required for each part of a PCR cycle will vary, depending on the nature of the template, $T_m$ of the primers, and length of the product A temperature of 94°C is adequate for most templates but some GC rich DNA or complex genomic DNA samples may require higher temperatures or longer incubation times The annealing temperature depends on the $T_m$ of the primers (**Note 3**), the elongation time at 72°C varies according to the length of the target being amplified, but most reactions allow 1 min for each kb of sequence The elongation time can be increased toward the end of a PCR reaction when the product concentration is high. In addition, a 5- to 10-min incubation at 72°C is attached to the end of most PCR programs to ensure complete elongation of products in the final cycle to maximize the final yield The number of cycles required will vary with the amount

of starting template. Too many cycles will give smearing and too few will give low yields

6 The addition of restriction sites is a useful application of PCR, but most restriction enzymes will not cleave sites close to the end of DNA fragments Addition of several GC bases can reduce this problem for enzymes, such as *Eco*RI, *Bam*HI, *Kpn*I, *Pst*I, *Sma*I and *Xba*I, by clamping the end as dsDNA Alternatively, the restriction site could be located internally in the primer, rather than at the 5' end

7 A range of template concentrations (1 pg–10 ng) can be tested to find the optimum that gives satisfactory yields with the minimum number of cycles of amplification Fewer cycles reduce the probability of misincorporation during DNA elongation, resulting in base changes to the final product (and subsequent clone)

8 The majority of heat-stable DNA polymerase enzymes have no proofreading activity, which can lead to PCR products containing sequence differences from the original template If high fidelity is required during the reaction, it is best to use one of the more recently developed enzymes, which have a proofreading activity In addition, it is advisable to minimize both the number of cycles performed and the length of template amplified Even if a proofreading enzyme is used, it is worthwhile checking the fidelity by sequence comparison of the product and the original template If the sequence of the template is unknown and it is being amplified by PCR to provide such data, it is advisable to sequence products from more than one reaction to obtain a consensus sequence

## References

1 Nicolas, O and Laliberte, J -F (1992) The complete nucleotide sequence of turnip mosaic potyvirus RNA *J Gen Virol* **73,** 2785–2793

2 Robertson N L , French, R , and Gray S. M (1991) Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses *J Gen. Virol* **72,** 1473–1477.

3 Frohman, M A , Dush, M K , and Martin, G R (1988) Rapid produciton of full-length cDNAs from rare transcripts amplification using a single gene-specific oligonucleotide primer *Proc Natl Acad Sci USA* **85,** 8998–9002

4 Foulds, I J , Lea, V J , Sidebottom, C , James, C M , Boulton, R. E , Brears, T , Slabas, A R , Jack, P L , and Stratford, R (1993) Cloning and sequence analysis of the coat protein gene of barley mild mosaic virus *Virus Res* **27,** 79–89

5 Lutke, H A , Chow, K C , Mickel, F S , Moss, K A , Kern, H F , and Scheele, G A (1987) Selection of AUG initiation codons differs in plants and animals *EMBO J* **6,** 43–48

# 29

# Antibody Production

**Carol Brattey and Robert Burns**

## 1. Introduction

Antibodies are produced by the immune systems of animals in response to the presence of foreign substances. Antibodies raised against regions of the viral coat protein (CP) (epitopes) can be exploited for virus detection, in 1977 Clark and Adams described the use of antibodies in ELISA to rapidly test a large number of plant sap samples for the presence of virus *(1)*. Antibodies produced against virus particles can also be used in Western blots (*see* Chapter 45) to assay CP expression in transformed plants *(2,3)*

Originally, all antibodies were derived from the serum of animals following immunization with the virus of interest. These polyclonal antibodies (PAbs) are essentially a heterogeneic mixture of molecules that will recognize several epitopes on the target protein The main advantage of PAbs is that their broad specificity may give a more robust test, perhaps allowing detection of most strains of a virus or even all members of a virus group. Paradoxically, the relative lack of specificity is also their main limitation, because they sometimes produce crossreactions with plant proteins that can give false positives in assays. Additionally, because they are produced on a batch basis, variable reactions may be encountered between bleeds from the same animal and between individual animals

In 1975, Kohler and Milstein reported that antibodies could be produced in tissue culture by creating recombinant cell lines from mouse myeloma cells and spleen cells from an immunized mouse *(4)* Antibodies made in this way are known as monoclonal antibodies (MAbs), because they are produced by cell lines derived from single recombinant parent cells

A good PAb is perfectly adequate for many purposes, but MAbs have the advantages of sensitivity, specificity, reproducibility, and consistent supply

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

from a defined source. Their main limitation is that the epitope to which they are raised is not conserved in all isolates of the virus, and false negatives result The two types of antibody should be considered complementary and both have a place in plant virology in which their different qualities can be exploited.

Production of high quality antibodies requires pure virus preparation for immunization. It is important that the isolates used are representative of the virus to be studied. In cases in which serological variation is reported, it may be prudent to use more than one isolate for immunization.

For PAb production, the virus preparation should be as free as possible from contaminating plant proteins; this is not so critical in MAb production, because crossreacting clones can be eliminated in the screening process. However, the purification should avoid severe physical or chemical damage, which may expose hidden epitopes, because it is possible that an antibody could be raised that will not detect native virus.

New Zealand white rabbits are normally used for production of PAbs because they yield high volumes of serum and can be easily bled from the marginal ear veins. High serum levels of specific antibody (approx 10 mg/mL) can be achieved by hyperimmunization. As the immunizations are carried out across several weeks, class-shifting of antibody type occurs; the resulting serum predominantly contains antibodies of class G, rather than the class M, which dominate during the primary response ***(5)***. Plant viruses are relatively immunogenic, but an increased response can be obtained by the use of an adjuvant. Preparations such as Quil-A or the Ribi adjuvant system encourage good titers of antibody, are easy to administer, and have fewer side effects than the Freund's formulations.

For MAb production, it is usual to use Balb-C mice, the strain from which most of the myeloma cell fusion partners were derived. Female mice are preferred, because they do not fight when kept together. Immunization protocols vary, but hyperimmunity is required to produce sufficient primed splenocytes Generally, the immune status of an animal is assessed by monitoring circulating antibody levels. Adjuvants can be used to improve the immune response, but should not be added to the final boost injection. Usually a batch of six mice are immunized, and the best responders are used for cell fusion (*see* **Note 1**)

Immunization and bleeding of animals is covered by legislation in most countries and must only be carried out by licensed personnel in licensed premises. The killing and removal of organs from mice is also subject to license in some countries, and it is usual that these procedures are carried out by animal technicians.

Although crude antisera preparations are useful for some purposes—e.g., for trapping virus particles on grids for electron microscopy—many serological techniques require the use of purified antibodies and antibodies that have

been modified with labels, often enzymes, which are attached by a simple conjugation process.

Before use in ELISA or blots, antibodies must be evaluated. It is desirable to test them for reactivity with other viruses, particularly closely related ones, for their reactions with healthy sap, and to optimize the dilutions to be used routinely. This should be done for each batch of antiserum (*see* **Notes 2** and **3**).

## 2. Materials

### 2.1. Buffers for Antibody Purification

Prepare solutions fresh and store at 4°C until required.

1 Phosphate-buffered saline (PBS), pH 7 4, 10X concentrate 80.0 g NaCl, 2.0 g $KH_2PO_4$, 29 0 g $NA_2HPO_4 \cdot 12H_2O$, 2.0 g KCl

Make up to 1 L with distilled water. The concentrate keeps well without the addition of sodium azide and can be diluted as required.

### 2.2. Media Required for Tissue Culture

1 Mixed thymocye medium (MTM) ***(6)***. This is required as a supplement for cells after fusion and for cloning (*see* **Note 4**) It is prepared by culturing the thymocytes of two different strains of rat in medium containing 15% fetal bovine serum (FBS) (Sigma, Dorset, UK) (*see* **Note 5**) for 48 h The medium is recovered from the cells by centrifugation and stored at –70°C until required
2 HAT medium Medium containing 15% FBS, hypoxanthine, aminopterin, and thymidine (HAT) (Gibco-BRL, Paisley, Scotland) is required for the selection of recombinant cells after cell fusion (*see* **Note 6**) It should be prepared in advance and stored at 4°C
3 RPMI-1640 (Sigma) RPMI-1640 containing 10 and 15% FBS is also required for routine tissue culture.

### 2.3. Consumables and Chemicals for Antibody Purification/Modification

1 Centriprep-30 and Centricon-30 concentrator tubes available from Amicon.
2 Alkaline phosphatase enzyme suspension in ammonium sulfate available from Sigma, Type VII
3 Mabtrap available from Pharmacia

## 3. Methods

### 3.1. PAb Production

1 Mix 200–500 µg of purified virus (*see* **Notes 7** and **8**) with an appropriate adjuvant to a final volume of 0 5 mL. Inject intramuscularly into the hind leg of the rabbit or subcutaneously into the neck scruff

2 Injections should be carried out once a week for 4 wk, and a test bleed should be taken 14 d after the last immunization
3. When the desired titer of specific antibody has been achieved, collection of blood can begin. Regulations concerning maximum volumes of blood that can be collected vary among countries and the appropriate authorities should be consulted
4 If large volumes of serum are required, then animals can be given boost injections after 6 wk and bleeding can recommence after 7–10 d
5 Blood should be allowed to clot at 4°C overnight and the serum should be collected by slow centrifugation

## 3.2. MAb Production

### 3.2.1. Immunization Protocol

1 Mix 50–100 μg of purified virus preparation (*see* **Notes 7** and **8**) for each mouse, with an appropriate adjuvant to a final volume of 100 μL in PBS This is injected intraperitoneally, or subcutaneously into the neck scruff
2 Injections should be carried out on d 0, 14, 28, and 56 Immune status should be checked by a test bleed 10 d after the last injection Blood can be obtained by performing tail-tip amputation under anesthesia with ether
3 Seventy-two hours before the cell fusion is performed, a final boost of 50–100 μg of virus should be given without adjuvant The injection site should be either intraperitoneal or intravenous If the intravenous route is to be used, then the amount of virus given should be reduced to approx 20 μg

### 3 2 2. Myeloma Cell Preparation

Several myeloma cell lines have been developed for monoclonal antibody production. The line must be hypoxanthine guanine phosphoribosyl transferase (HGPRT) defective, so that it is sensitive to aminopterin, and must not secrete its own antibody (*see* **Note 6**). The enzyme deficiency is required so that only recombinant cells can grow in the HAT medium as they inherit the necessary enzyme pathway from the parent spleen cell. Cell lines derived from the NS1 myeloma, such as NS 0 *(7)*, are ideal for MAb production.

Culture the cells in a 37°C incubator with 5% $CO_2$ and 90% humidity for several days prior to cell fusion, at subconfluent numbers to ensure log growth Myeloma cells are fairly undemanding and can be grown in RPMI-1640 medium containing only 5% FBS.

### 3.2.3 Preparation of Spleen and Myeloma Cells for Fusion

The immunized mouse is killed by cervical dislocation and the spleen removed aseptically. The spleen is placed in a sterile 25-mL universal container containing sterile PBS and is kept at 4°C. The splenocytes are obtained by homogenizing the spleen either with a manual tissue homogenizer or by grinding between two frosted-glass slides. Suspend the cells in PBS and wash

by centrifugation at 250*g*. Resuspend in 10 mL of PBS and perform a viable cell count (*see* **Note 9**)

Harvest the myeloma cells from the tissue culture flasks by removing the medium and replacing it with PBS containing 0.02% (w/v) ethylenediamin-etetra-acid (EDTA). Once they have detached from the surface of the flasks decant into universal containers and wash by centrifugation at 150*g*. A viable cell count should be performed to estimate numbers (*see* **Note 9**)

### *3.2.4. Cell Fusion (Modification of* ***ref.*** *8)*

Keep all media in a water bath or incubator set at 37°C.

1. Place 26 × $10^6$ each of spleen and myeloma cells together in a universal container
2. Pellet cells at 125*g* for 5 min and pour off PBS
3. Tap pellet gently to loosen cells
4. Add 1 mL of 50% polyethylene glycol 4000 (Fisons, Leicestershire, UK) (PEG)/RPMI-1640 medium (no FBS)
5. Resuspend cells gently
6. Pellet cells at 175*g* for 5 min
7. Add 5 mL of RPMI-1640 medium (no FBS) do not disturb the pellet
8. Gently resuspend
9. Pellet cells at 175*g* for 5 min
10. Pour off supernatant and slowly add 5 mL HAT medium with 15% FBS
11. Leave pellet for 7 min, then gently resuspend.
12. Place 0.1 mL of HAT medium with 15% FBS and 15% MTM medium into each of the 96 wells of a tissue-culture plate. Add 0 1 mL of the cell suspension from **step 11**.
13. Incubate the cells in a 37°C incubator with 5% $CO_2$ and 90% humidity for 7 d, and then assess visually for colony growth Each well should have 0.1 mL of HAT medium with 15% FBS added. When cell colonies are one-third to one-half confluent in the wells, the tissue culture supernatant should be assayed (*see* **Notes 2** and **3**) for the presence of the antibody of interest (*see* **Subheading 3.5.**, *see* **Note 10**)
14. Cells that are producing the desired antibody should be cloned by limiting dilution (1 cell/well) until stability and clonality can be ensured
15. Keep stocks of cells in liquid nitrogen so that MAbs can be produced at a later date
16. Bulk cultures of cells can be grown to produce mg quantities of MAb. Cell lines produce 10–50 mg/L of medium, which can then be purified by affinity chromatography on protein A or G (Pharmacia, St Albans, UK)

## *3.3. Purification of PAb*

In **steps 2–3**, work in a small, clean beaker on a magnetic stirrer set at a slow speed at room temperature

1 Add 9 mL distilled water to 1 mL of crude antiserum
2 Slowly add 10 mL neutralized saturated ammonium sulfate (Sigma) while stirring (*see* **Note 11**).
3 Remove from stirring and leave to precipitate for about 1 h at room temperature The resulting solution should appear viscous and cloudy
4 Centrifuge for 15 min at 9000*g* and retain precipitate
5 Dissolve the precipitate in 2 mL of half-strength PBS
6 Add the dissolved precipitate to a Centriprep tube, top up to the mark with half-strength PBS and spin at 9000*g* (speed not critical) for 20 min. Discard the liquid that has drained into the central part of the tube Replace with half-strength PBS up to the mark and repeat twice more, to remove traces of ammonium sulfate
7 Measure the optical density at a wavelength of 280 nm (*see* **Note 12**) and adjust by dilution in half-strength PBS until the reading is 1 4 (this dilutes the γ globulin to 1 mg/mL) The solution can be concentrated by further spinning in the Centriprep tube, if the reading is <1 4
8 Aliquot to volumes suitable for storage (1 mL is often convenient) and store at –20°C, or lyophilized Sodium azide (Sigma) may be added to 0 02% w/v as a preservative (*see* **Note 13**)

### 3.4. Preparation of Antibody–Enzyme Conjugate (Using Alkaline Phosphatase Suspension in Ammonium Sulfate)

1 Centrifuge suspension containing 5000 U of enzyme to precipitate (3 min at high speed in a microcentrifuge)
2 Dissolve precipitate in 2 mL purified γ-globulin (1 mg/mL, prepared as above)
3 Add to a Centricon tube and centrifuge at 9000*g* for 20 min (*see* **Note 14**) Replace with half-strength PBS up to the 2-mL mark, and repeat twice more, to remove traces of ammonium sulfate
4 Add fresh gluteraldehyde (Sigma) to 0 05% (it may be convenient to prepare a 5% gluteraldehyde solution in distilled water and add 20 µL to the 2 mL of antibody solution) Mix well
5. Leave for 4 h at room temperature or overnight at 4°C A faint brown color may develop
6 Repeat **step 3** to remove traces of gluteraldehyde Final volume should be 2 mL
7 Add bovine serum albumin (Sigma) (BSA) to 5 mg/mL w/v, mix to dissolve, and store at 4°C
8 Sodium azide may be added to 0 02% w/v, to enhance storage life (*see* **Note 13**)

### 3.5. Screening and Evaluation of Antibodies

Before using antibodies in an assay, their efficacy should be tested against the virus or virus group concerned, and working dilutions optimized. They should be screened against large panels of virus isolates and a range of cultivars of healthy plants. It is important to include closely related viruses, to ensure that crossreactivity does not occur In the case of MAbs, early screening

will allow clones secreting antibodies of undesired specificity to be discarded. Until the specificity of an antibody is established, it is of little use for differential diagnosis.

Dilutions of purified antibody (1 mg/mL) in the range of 1:250–1.4000 should be tested against dilutions of purified virus or sap from infected plants diluted 1.10–1:1000 in buffer. Selection of working dilutions should be chosen by the ratio of reaction of known positive material relative to the negative control (*see* **Notes 2** and **3**). The combination of antibody, virus, and sap dilution showing the highest signal to background should be chosen.

## 4. Notes

1 Mice should be permanently marked to allow identification at a later date This is usually done by ear-punching or tattooing, but electronic tagging devices can be used

2. It is important that the assay system used at this stage should be the same as the test system for which the antibody is intended Antibodies assayed by ELISA, for example, do not always perform well in western blots or immunofluorescence assays This is because some epitopes, when blotted or electrophoresed, lose the three-dimensional structure required for antibody recognition, and this is particularly important when using MAbs.

3 It is vital that whenever antibodies are used, appropriate controls (infected and healthy plant tissue) are included in the assay, to reduce the risk of false-positive or false-negative results The negative control should be of the same type, preferably the same variety, of plant as used in the assay

4 Some workers use feeder layers of peritoneal macrophages to provide the necessary cytokines to support hybridoma survival after cell fusion or cloning MTM *(6)* performs the functions of feeder cells and supports the growth of primary hybridomas and cloned cells It is easy to use, can be stored frozen for long periods of time, and can be assessed for sterility when first made

5 Batches of FBS *(5)* vary in their ability to support cell growth and should be assessed by cell-cloning assays prior to purchase Most suppliers will provide small quantities for assessment and reserve the required amount until cell growth studies have been performed.

6 Other enzyme-deficient cell lines have been developed for hybridoma production, and some workers report that they are better than HAT-sensitive lines

7 It is not important for antibody production if, at this stage, the virus particles are a little aggregated or broken.

8 For the purpose of antibody production, do not add sodium azide to the purified virus. If mercaptoethanol or other noxious substance has been used in the antigen purification, this can be removed by dialysis or by using Centricon ultracentrifugation tubes.

9. Viable cell counts can be carried out either by trypan blue exclusion in visible light or by using acridine orange–ethidium-bromide in UV light The acridine

orange–ethidium-bromide method is easier to assess

10 Before making final selection of MAbs, the isotype of the antibodies should be determined using the Sigma immunotype kit If possible, antibodies of class M and A should be discarded, because they are difficult to purify. Antibodies of the class G3 should be avoided, because they often aggregate spontaneously after purification

11 It is very important that the ammonium sulfate solution has been adjusted to pH 7 0 by the addition of HCl before use in this step

12 If sodium azide is to be added as a preservative, it is important that this is not included until after the OD reading has been taken and the dilution assessed, because azide affects the OD reading at this wavelength.

13 **Caution:** Sodium azide is very toxic and can form explosive compounds with metals Solutions containing azide should be labeled as such and handled and disposed of with care

14 Centricon tubes fit neatly inside Centriprep tubes with the insert removed Centripreps can be used as adapters to spin Centicons in a rotor designed for 50-mL tubes

## References

1 Clark, M F and Adams, A M (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses *J Virol Methods* **34,** 475–483

2 Ehlers, U , Commandeur, U , Frank, R , Landsmann, J , Koenig, R , and Burgermeiser, W. (1991) Cloning of the coat protein gene from beet necrotic yellow vein virus and its expression in sugar beet hairy roots *Theor Appl Genetics* **81,** 777–782

3 Fang, G and Grumet, R (1993) Genetic engineering of Potyvirus resistance using constructs derived from the Zuccini Yellow Mosaic virus coat protein gene *Mol Plant–Microbe Interact* **6, 3,** 358–367.

4 Kohler, G and Milstein, C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity *Nature* (London) **256,** 495–497

5 Roitt, I , Brostoff, J , and Male, D (1985) *Immunology* Churchill Livingstone, International, Edinburgh, pp 12 1–12 3

6 Micklem, L. R , McCann, M. C , and James, K (1978) The use of rat mixed thymocyte culture-conditioned medium for hybridoma production, cloning and revival *J Immunol Methods* **104,** 81–86

7 Galfre, G and Milstein, C (1981) Preparation of monoclonal antibodies strategies and procedures *Methods Enzymol* **73,** 3–46

8 Kennet, R H , Denis, K A , Tung, A S , and Klinman, N R (1978) Hybrid plasmacytoma production fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells *Curr Top Microbiol Immunol* **81,** 485

# 30

# Expression Library Screening

**Gary D. Foster**

## 1. Introduction

When trying to identify a clone within a cDNA library, which may contain a coat protein (CP) gene, one useful technique may by immunological screening, using antibodies raised against either purified virus or isolated CP. Antibody screening can be carried out on a cDNA cloned into a wide range of vectors, including plasmids and phage-based vectors. Indeed, a whole plethora of commercial vectors are now available that have been optimized for generating expression libraries, including λ-gt11, λZAP (Stratagene, La Jolla, CA). However, antibody screening can be carried out on the simplest of plasmid vectors, based on the principle that, if the plasmid uses blue/white color selection, then a percentage of the cDNA inserts will be expressed as a fusion protein with β-galactosidase when the cells are induced with IPTG. The method described within this chapter will deal with such a plasmid screen, with readers directed to λ-screening chapters by Somssich and WeiBhaar in *Plant Gene Isolation* ***(1)*** and Hurst in *cDNA Library Protocols* ***(2)***, and (one of the original and best descriptions) by Huynh et al. in *DNA Cloning A Practical Approach* ***(3)***, all being good references for suitable lambda protocols. A typical immunological screen is shown in **Fig. 1**, for a pUC13 vector ***(4)***. Double-stranded cDNA to the carlavirus, Helenium virus S (HelVS) was ligated into *Sma*I digested pUC13 vector and transformed into competent *Escherichia coli* Colonies were screened with both nucleic acid probes using HelVS specific ($^{32}$P) first-strand cDNA (**Fig. 1A**) and also using HelVS polyclonal antisera (**Fig. 1B**). Two clones at positions 19 and 23, designated pHel19 and pHel23, were detected using polyclonal antisera. Both clones were further analyzed as to the possible size of the CP-coding region that was being expressed by polyacrylamide gel electrophoresis (PAGE) and Western analysis of total

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

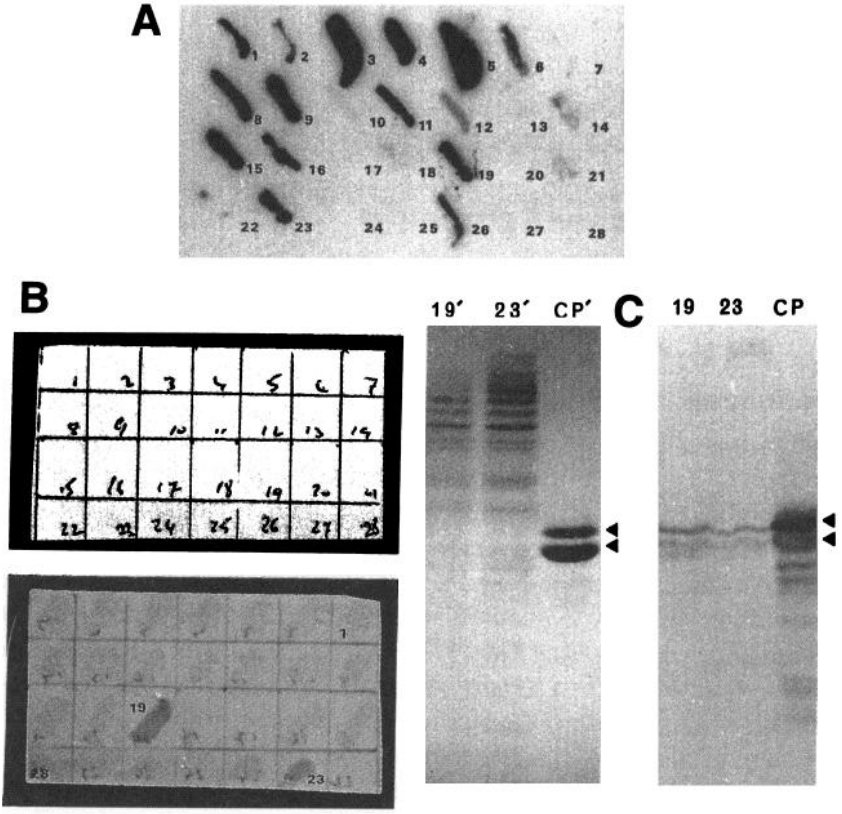



Fig. 1. **(A)** Colony hybridization using HelVS specific ($^{32}$P) cDNA. Position 28 represents pUC 13 nonrecombinant control. **(B)** Colony hybridization using HelVS polyclonal antisera. Colonies were streaked onto nitrocellulose (top panel) and grown overnight prior to screening. Note that color development is evident on the reverse side on which colonies were streaked (bottom panel). Colony 28 represents pUCl3 nonrecombinant control. **(C)** Western blot analysis of clones expressing HelVS coat protein. Lanes 19', 23', and CP': Coomassie blue stained gel of bacterial lysates and HelVS CP. Lanes 19, 23, and CP: Western blot analysis of bacterial lysates and HelVS CP reacted with HelVS polyclonal antisera. Positions of the CP-related products are indicated with arrows. No signals were obtained in Western blots from untransformed bacterial cell lysates.

cell protein from bacterial lysates. As shown in **Fig. 1C**, both pHel19 and pHel23 revealed protein bands similar in size to that found for HelVS viral CP. Both clones were subsequently sequenced and confirmed to be the CP gene of HelVS *(5)*.

## 2. Materials

### 2.1. Library Plating

1 Isoproply β-D-thiogalactopyranoside (IPTG) 1*M* stock in water Store at –20°C
2 Suitable bacterial host.
3 Nitrocellulose filters (S&S nitrocellulose BA85).
4 Suitable solid-growth-media plates (containing the appropriate antibiotic for the vector in use)

### 2.2. Screening for Plasmids Expressing CP

1 Bug-busting buffer: 2X SSC containing 0.5% sodium dodecyl sulfate (SDS)
2 Tris-buffered saline (TBS) 50 m*M* Tris-HCl, pH 7.5, 200 m*M* NaCl
3 Blocking buffer 5% dried milk powder in TBS
4 TBST: TBS containing 0 05% Tween-20
5 High titer polyclonal primary antibody or suitable cocktail of monoclonal antisera
6. Suitable secondary conjugated antibody for detection
7. Suitable radiochemical or chromogenic reagent of your choice

### 2.3. Western Blot Analysis of Expressed Fusion Proteins

1 SDS-PAGE loading buffer 2% SDS (w/v), 5% mercaptoethanol (v/v), 10 glycerol (v/v) in 0 125 m*M* Tris-HCl, pH 6.8

## 3. Methods

### 3.1. Library Plating

1 Transform cDNA library into a suitable bacterial host and spread out on plates at a suitable density to visualize single colonies and incubate overnight at 37°C.
2 Draw and number a grid on a Petri-dish-sized nitrocellulose circle, soak in 10 m*M* IPTG, and allow to air-dry before carefully placing it onto the surface of a solid media plate.
3 Draw and number a similar grid on the base (not the lid) of another solid media plate
4 Pick individual colonies from the original cDNA library plate described in **step 1**; streak onto a numbered position on the nitrocellulose grid, and streak onto the same position on the plain agar master plate, using a sterile loop (*see* **Note 1**)
5 When a suitable number of colonies have been streaked out onto both plates, invert the plates and incubate overnight at 37°C

### 3.2. Screening for Plasmids Expressing CP

1 Remove filters and place colony-side up on filter paper (e g , Whatman 3MM paper), presoaked in Bug-busting buffer, for 2–3 min (*see* **Note 2**). Remove and immediately go to **step 2**
2. Incubate the filters in blocking buffer overnight at room temperature with gentle agitation (*see* **Note 3**)

3 Wash the filters five times in TBST, 5 min for each wash with gentle agitation (*see* **Note 4**)
4 Incubate the filters for 90 min at room temperature, with gentle agitation, in an appropriate dilution of primary-CP antibody (*see* **Note 5**), diluted in fresh blocking buffer
5 Wash the filters five times in TBST, 5 min for each wash with gentle agitation (*see* **Note 4**)
6 Incubate the filters with secondary antibody conjugated with a suitable detection system (*see* **Note 6**) (e g , alkaline phosphatase) for 90 min at room temperature with gentle agitation
7 Wash filters as in **step 5**
8 Develop the filters using a suitable detection system, e g , color detection or chemiluminescence (*see* **Note 6**)

### 3.3. Western Blot Analysis of Expressed Fusion Proteins

1 Grow antibody-positive colonies overnight in liquid broth at 37°C in the presence of 5 mmol/L IPTG
2 Harvest cells (0 5 mL) by microcentrifungation and resuspend in SDS-PAGE loading buffer (50–100 µL) and boil for 2 min
3 Spin briefly in a microcentrifuge to remove cell debris and remove supernatant to a fresh tube
4 Boil sample for 2 min prior to separation on 12 5% polyacrylamide gels, Western blotting onto a suitable membrane and detection of bands as described above (*see also* Chapter 45)

## 4. Notes

1 An alternative to using sterile loops is to use autoclaved pipet tips, using a fresh tip after each duplicate streak has been completed
2 We have observed that good signals can be obtained using no pretreatment of the filters However, best signals are obtained when the colonies are treated to disrupt them, such as bug-busting buffer or by treating the colonies as described for nucleic acid probes
3 Gentle agitation can be achieved using a shaking platform, a shaking incubator (set at room temperature), a rotating platform, or gentle shaking of the container by hand every 5–10 min
4 If multiple filters are screened at the same time, ensure that the filters do not stick together during incubation and shaking.
5 A typical dilution to use for good-quality polyclonal antisera would be a 1·500 or 1 1000 dilution
6 Commercial antibodies are available that are coupled to enzymes such as horseradish peroxidase or alkaline phosphatase that react with specific determinants on primary antibodies (species specific) These primary–secondary complexes can then be detected using chromogenic substrates, available from a wide range of commercial sources, which also provide clear instruction protocol booklets (e g , Stratagene, Amersham, Boehringer Mannheim, Promega, and Pharmacia)

## References

1 Somssich, I E and WeiBhaar, B (1996) Expressing library screening, in *Plant Gene Isolation* (Foster, G D and Twell, D., eds ), Wiley, New York
2 Hurst, H. C (1997) Immunological screening of phage cDNA expression libraries, in *cDNA Library Protocols* (Cowell, I G and Austin, A. A., eds ), Humana, Totowa, NJ, pp 155–159
3 Huynh, T V, Young, R A , and Davies, R. W. (1988) Constructing and screening cDNA libraries in lambda-gt10 and lambda-gt11, in *DNA Cloning A practical Approach*, vol I (Glover, D M , ed.), IRL, Oxford, UK, pp 49–78
4 Foster, G D., Scott, R , Draper, J , and Mills, P R (1992) Expression of Helenium virus S coat protein in Escherichia coli, in vitro in rabbit reticulocyte and transgenic tobacco *Acta Virol* **36,** 567–575
5 Foster, G D , Millar, A W , Meehan, B M , and Mills, P R (1990) Nucleotide sequence of the 3-terminal region of Helenium virus S RNA *J Gen Virol* **71,** 1877–1880

# 31

# In Vitro Transcription and Translation

**Roisin Turner and Gary D. Foster**

## 1. Introduction

Large amounts of active mRNA can be synthesized in vitro. In vitro-transcribed RNA molecules may subsequently be used for various purposes, e.g., in vitro translation. Several plasmids are available that are used to produce transcript molecules from cloned DNA inserts These contain one or more promoter sequences, recognized by the T3, T7, or SP6 RNA polymerase enzymes, flanking the multiple-cloning site. Once cloned, an insert, e.g., a DNA sequence coding for a viral coat protein (CP), may be transcribed into mRNA in a simple reaction exploiting these enzymes. It is generally thought that a mRNA molecule must contain a 7-methyl guanosine or cap structure at the 5' end to be efficiently translated *(1)*, but we have found that sufficient quantities of proteins are generated for analysis from uncapped transcripts, at least in vitro.

In vitro translation provides a means of synthesizing proteins from mRNA in a microcentrifuge tube. Reactions are carried out in cell lysates, which contain all the macromolecular components necessary for protein synthesis, e.g., ribosomes, translation factors, tRNAs, and amino acids. Lysates are produced by disrupting the cells and removing the cell debris by centrifugation.

Two of the most useful translation systems are derived from rabbit reticulocytes *(2)* and from wheat germ *(3)*. Both systems provide a reliable, convenient system to initiate translation and produce a full-sized polypeptide Rabbit reticulocyte lysate (RRL) is favored for translation of larger mRNA molecules, wheat-germ lysate (WGL) is favored when low amounts of double-stranded RNA or oxidized thiols are present, which are inhibitory in RRL. Systems derived from *Escherichia coli* are also widely used *(4)*

Artificial translation systems are further supplemented with a chemical method of producing energy, phosphocreatine kinase, and phosphocreatine.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

Additional tRNAs are also supplemented to expand the range of RNAs that are translated. Hemin may also be added to prevent inhibition of translation initiation, because it suppresses an inhibitor of the elongation initiation factor eIF-2a. Potassium and magnesium are also added to a level recommended for translation of most mRNA species. Lysates are treated with micrococcal nuclease to remove any traces of endogenous mRNA that could interfere with subsequent reactions.

Once the translation system has been programmed with the transcript of interest, proteins produced can be detected by incorporation of a radiolabeled ribonucleotide, generally [$^{35}$S] methionine or cystine, or [$^{3}$H]leucine. Products may be visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. This allows proteins to be sized by comparison with predetermined markers. Being an in vitro system, smaller protein bands are usually present on autoradiographs, caused by premature termination of translation by lysate ribosomes. If specific antibodies are available against the product of an in vitro translation reaction, a further means of identifying and quantifying may be provided; e.g., immunoprecipitation protocols may be employed.

Recently, coupled transcription–translation systems have become available in which circular DNA can be used as template, thereby greatly reducing the workload. Combination systems that consist of a mixture of RRL and WGL may also be used.

## 2. Materials

### 2.1. In Vitro Transcription

1. Transcription buffer, usually supplied with the polymerase enzyme being used at either a 10X or 5X concentration (e.g., Promega [Southampton, UK] 5X buffer supplied with the T3 RNA polymerase, *see* **Note 1**)
2. Ribonucleotides diluted to a 10-m*M* concentration (e.g., Pharmacia [Herts, UK] ribonucleotides supplied at 100 m*M*)
3. Dithiothreitol (DTT) at 100 m*M*
4. RNase inhibitor (e.g., Promega RNasin®, supplied at 33 U/μL)
5. DEPC-treated water (*see* **Note 2**)
6. RNA polymerase enzyme (e.g., Promega T3 RNA polymerase supplied at 5 U/μL)

### 2.2. In Vitro Translation

1. Commercial in vitro translation system (e.g., Promega RRL or WGL translation systems, *see* **Note 3**).
2. Ribonuclease inhibitor (e.g., Promega RNasin®, supplied at 33 U/μL)
3. [$^{35}$S] Methionine (1200 Ci/mmol) at 10 mCi/mL (e.g., Amersham, Arlington Heights, IL)
4. DEPC-treated water

### 2.3. Quantification of Radiolabeled Amino Acid Incorporation

1 Whatman GF/A glass fiber filters (Maidstone, UK)
2 1*M* NaOH, 2% $H_2O_2$
3 Ice-cold 25% trichloroacetic acid (TCA), 2% casamino acids, vitamin assay grade (Difco, Detroit, MI)
4 5% ice-cold TCA
5 Acetone
6 Scintillation fluid (e g , Opti-flur O, Packard)

### 2.4. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1 Gel apparatus (e g , Bio-Rad vertical minigel apparatus)
2 10X Glycine running buffer (Tris-glycine, pH 8.3)· For 1 L mix 30 g Tris-base, 144 g glycine, 100 mL 10% SDS, then add distilled water to volume
3. Acrylamide/*bis*-acrylamide solution (30.8%) For 100 mL, dissolve 30 g acrylamide and 0 8 g *bis*-acrylamide in distilled water Store at 4°C
4. 1 5*M* Tris-HCl, pH 8 8 For 1 L, dissolve 181 65 g of Tris-base in 800 mL of distilled water, and titrate to the correct pH with concentrated HCl Make to 1 L with distilled water
5 0 5*M* Tris-HCl, pH 6 8 For 1 L, dissolve 60 55 g of Tris-base in 880 mL of distilled water, titrate to the correct pH with concentrated HCL Make to 1 L with distilled water
6 10% SDS
7 Tetramethylethylene diamine (TEMED).
8 1% Ammonium persulfate (make fresh before use)
9 Propanol water (1.1)
10 Sample loading buffer· 2% (w/v) SDS, 5% β-mercaptoethanol, 10% glycerol, 0 01% bromophenol blue, in 0 125*M* Tris-HCl, pH 6 8
11 Coomassie blue stain solution Distilled water, methanol, and glacial acetic acid at a ratio of 5.5 1, with 0 1% Coomassie blue
12 Destain solution As in **item 11**, without the Coomassie blue

### 2.5. Autoradiography

1 Gel-drying apparatus
2. X-ray film (e.g , Kodak's X-omat AR or Amersham's Hyperfilm)

### 2.6. Immunopreciptation

1 8% SDS
2 TNTE buffer 1% Triton-X 100, 150 m*M* NaCl, 2 m*M* EDTA, 50 m*M* Tris-HCl, pH 7.8
3 High-salt TNTE 1% Triton-X 100, 0 95*M* NaCl, 2 m*M* EDTA, 50 m*M* Tris-HCl, pH 7 8
4 Protein A Sepharose (Sigma)
5 Sample loading buffer (*see* **Subheading 2.4., item 10**)

## 3. Methods

### 3.1. In Vitro Transcription

Once the gene of interest has been cloned into the appropriate vector, template DNA must be linearized at a restriction site downstream from the insert that is to be transcribed. After digestion, all traces of RNases and other proteins should be removed. This is achieved by extracting with phenol and chloroform, and precipitating with ethanol. Treatment with proteinase K prior to phenol extraction is optional (*see* **Note 4**). The DNA pellet obtained is resuspended in DEPC-treated water and generally a small sample is checked on an agarose gel to ensure digestion has been successful.

Detailed instructions regarding assembly of the in vitro transcription reaction are supplied with the various kits that are available, but a summary will be provided here

1. The following components are mixed in a 1.5-mL microcentrifuge tube 1–2 µg of linearized DNA, 2 µL of RNA transcription buffer (10X); 20–40 U RNase inhibitor, 1 µL each of rATP, rCTP, rGTP, rUTP (10 m*M* stock), 1–10 U RNA polymerase (T7, T3, SP6) (*see* **Note 5**), DEPC-treated water to a final volume of 20 µL
2. Reactions are incubated at 37°C for 30–60 min
3. DNA template is removed by adding 2 U of RNase free DNase and incubating at 37°C for a further 15 min
4. Transcripts are then purified by phenol·chloroform extraction and ethanol precipitation
5. Purified RNA can be quantified spectrophotomically or visually on an ethidium bromide-stained agarose gel mRNA molecules produced may now be used in translation reactions

Capping increases the stability of a transcript molecule Capped transcripts can be readily synthesized in vitro by the addition of cap analog to the transcription reaction. The level of normal GTP in the reaction is reduced to a concentration of 50 µ*M*, and cap analog (m7G[5']ppp[5']G) is added at a concentration of 10 m*M* The reaction is carried out according to **Subheading 3.1.**

### 3.2. In Vitro Translation

Translation reactions are carried out according to manufacturer's instructions provided with RRL or WGL. Transcript should be heated to 65°C before use, to increase the efficiency of translation by removing secondary structure. Components of the reaction are assembled in a 1.5-mL microcentrifuge tube. Rabbit reticulocyte lysate reactions are incubated at 30°C, and wheat-germ lysate reactions at 25°C, for 60 min. Results may be analyzed by incorporation assays (**Subheadings 2.3.** and **3.3.**) or by SDS-PAGE (**Subheadings 2.4.** and **3.4.**).

### 3.3. Quantification of Radiolabeled Amino Acid Incorporation

1 2 µL of the completed translation reaction is removed and is added to 98 µL of 1*M* NaOH/2% $H_2O_2$ in a 1 5-mL microcentrifuge tube
2 After vortexing, the tube is incubated at 37°C for 10 min.
3 Translation products are precipitated by adding 900 µL of ice-cold 25% TCA/2% casamino acids and incubating on ice for 30 min
4 250 µL of this TCA–reaction mix is precipitated onto a Whatman GF/A glass fiber filter by vacuum filtration
5 The filter is washed three times with 2 mL of ice-cold 5% TCA and once with 2 mL of acetone. The filter is then allowed to dry completely at room temperature or under a heat lamp
6 The filter is placed in 2 mL of scintillation fluid, and a value for incorporated counts, given as counts per million (cpm), is obtained in a liquid scintillation counter, on the appropriate setting for the chosen isotope
7 To determine the total counts present in the reaction, a 5-µL aliquot of the remaining TCA–reaction mix is spotted directly onto a separate glass filter. The filter is allowed to dry and a value for total counts is determined, as for incorporated counts in **step 6** (*see* **Note 6**)
8 Percentage radiolabeled amino acid incorporation is determined as cpm of washed filter/cpm of unwashed filter × 50

### 3.4. SDS-PAGE

1 Glass plates are cleaned, first with detergent and then with ethanol The gel apparatus is then assembled
2 Constituents of the resolving gel are mixed in a beaker These will vary according to the concentration and volume of the gel, but constituent amounts for a 12 5% SDS-PAGE gel in a Bio-Rad vertical minigel apparatus will be provided (20 mL total volume) (*see* **Note 7**)
   a 6 5 mL Sterile distilled water
   b 5 0 mL 1 5*M* Tris-HCl, pH 8 8
   c 8.0 mL Acrylamide *bis*-acrylamide (30.0.8%)
   d 0 2 mL 10% SDS
3. Immediately prior to pouring, 50 µL of 10% ammonium persulfate and 5 µL of TEMED are added
4 The solution is poured between the two glass plates, leaving enough space for the well former and stacking gel A fine layer of either water or propanol/water is overlayed, and the gel is allowed to polymerize for 60 min
5 Components for the stacking gel are assembled in a beaker
   a 3 05 mL Sterile distilled water
   b 1.25 mL 1.5*M* Tris-HCl, pH 8.8.
   c 0 68 mL Acrylamide.*bis*-acrylamide (30 0 8%)
   d 0.05 mL 10% SDS
6. The propanol–water or water layer is poured from the polymerized resolving gel 25 µL of ammonium persulfate and 2 5 µL of TEMED are added to the stacking

gel, and the mixture is immediately poured between the two glass plates on top of the resolving gel, with the well-forming comb in place The gel is allowed to polymerize for 30 min

7 When the gel is ready, remove the well comb and place in the electrophoresis apparatus Fill with 1X glycine running buffer
8 To 10 µL of translation reaction, add 10 µL of SDS loading buffer and boil for 2 min to denature proteins
9 Load sample onto the gel and electrophorese at voltages between 100 and 200 V, until the bromophenol blue dye in the sample buffer has disappeared into the running buffer
10 The gel is then removed from the apparatus and proteins are stained in Coomassie blue stain for 30 min, and destained for 30 min. This fixes the proteins into the gel
11. The gel is then dried and exposed to X-ray film (*see* **Note 8**)
12. The size of the in vitro translation products may then be compared with mol-wt markers (radioactive or nonradiocative) run on the same gel (*see* **Note 9**)

### 3.5. Immunoprecipitation

1 From a typical reticulocyte translation of 50 µL vol, remove 40 µL of the translation reaction to a fresh microcentrifuge tube (save remaining 10 µL as a unprecipitated control), and add 14 µL of an 8% SDS solution, and mix by inverting
2 Heat the tube at 90°C for 2 min
3 Add 540 µL of the TNTE buffer and mix gently by inverting the tube several times; allow to cool to room temperature
4 Add 2 µL of the required antibody and mix the tube by inverting gently
5 Incubate the tube at 20°C for 2 h, or 4°C overnight, on a rotation platform
6 Add 2 5 mg of protein A Sepharose
7 Attach to a rotating platform for 1 h at 20°C
8 Centrifuge the tube at 1600*g* in a microcentrifuge for 5 min to collect the pellet
9 Remove all supernatant and wash the pellet once in TNTE and collect the pellet by microcentrifugation
10 Wash the pellet twice in High-salt TNTE, as in **step 9**
11 Wash the pellet four times in TNTE, as in **step 9**
12 Resuspend the final pellet in 20 µL of sample loading buffer, heat to 90°C for 2 min before loading onto SDS-polyacrylamide gels alongside the unprecipitated control from **step 1**

## 4. Notes

1 Transcription buffer, either at a 5X or 10X concentration, is supplied with the polymerase enzyme used This buffer provides the optimal conditions for enzyme activity 10X buffer for T3 RNA polymerase consists of the following components. 200 m*M* Tris-HCl, pH 8 0, 40 m*M* $MgCl_2$, 10 m*M* spermidine, 250 m*M* NaCl

2 Every precaution is taken to prevent contamination from RNases All solutions and apparatus are treated with diethylpyrocarbonate (DEPC)
3 In vitro translation mixtures are stored at –70°C for short periods of time, and under liquid nitrogen for long periods.
4. Contaminating RNases may be removed from the linearized DNA template used for in vitro transcription by treatment with proteinase K (100 µg/mL) and 0 5% SDS for 30–60 min at 37°C before phenol:chloroform extraction
5. The polymerase enzyme is unstable and must always be stored on ice Concentrations of enzyme toward the upper scale of that stated in **Subheading 3.1.**, that is, 5–10 U/20-µL reaction, provide the best results
6 Values for incorporation of radiolabeled nucleotide between 2 and 10% should be expected
7 SDS-PAGE gel solutions may be degassed for 1–2 min before adding the ammonium persulfate and TEMED
8 Exposure times vary, depending on radioactive label and quantity of RNA used to prime translation reactions, however, typical times are from overnight to 2–3 d.
9 A translation product obtained from a potential CP clone, which is of similar size to the CP obtained from purified virus particles, is a good indication that the clone is of full length and there are no artifically introduced frameshifts or stop codons. However, further evidence, such as immunopreciptation of the translation product with antisera against the CP, is required (*see* **Subheading 3.5.**)

## References

1 Shatkin, A J (1976) Capping of eukaryotic mRNAs *Cell* **9,** 645–653
2 Pelham, H R B and Jackson, R J (1976) An efficient mRNA-dependent translation for reticulocyte lysate *Eur J Biochem* **67,** 247–256
3 Roberts, B E and Patterson, B M (1973) Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell free system from commercial wheat germ. *Proc Natl Acad Sci USA* **70,** 2330–2334
4 Lengyel, P and Soll, D. (1969) Mechanism of protein synthesis *Bacteriol Rev* **33,** 264–301

# 32

# Analysis of Coat Protein Expression Cassettes in Protoplasts

**Jonathan H. Weston and Roisin L. Turner**

## 1. Introduction

Transient gene expression studies of plant virus genes or parts of genes can be studied using plant protoplasts. Such studies are dependent on a number of important factors:

1. The method for isolating plant cells or protoplasts Methods are now well-established for the isolation of protoplasts from a wide range of plant species *(1)*
2. Vector requirements or a gene construct that will be expressed in the protoplast under the culture conditions It is important to use a vector that enables the expression of the insert gene Genes lacking a plant promoter are expressed using the cauliflower mosaic virus (CaMV) 35S gene promoter. CaMV, one of the best-characterized plant DNA viruses, was used in early plant transformation studies, and the CaMV 35S promoter is recognized in a wide range of plants For this reason, it is the most commonly used promoter element to express foreign genes during transformation Gene expression cassettes consist of a multiple cloning site placed between a CaMV 35S promoter, or other strong promoter, and a poly(A) addition site or nopaline synthase (nos) terminator *(2,3)*. Inserting the gene of interest into the multiple cloning site will drive the expression and allow transient studies
3. An efficient method for introducing the nucleic acid into the isolated cells A number of successful techniques exist in order to transfect nucleic acid into protoplasts, such as polyethylene glycol (PEG) treatment, electroporation, microinjection, and particle bombardment PEG is a more common choice for protoplast transformation, because it does not require more specialized equipment associated with electroporation or biolistics The precise function that PEG has during transfection is unknown, however, it is believed that PEG precipitates nucleic acid onto the protoplast plasma membrane and then stimulates its uptake by endocytosis *(4)*

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

4 A sensitive method for the detection of gene expression Detection of gene expression depends on the nature of the insert itself. For example, if a reporter gene has been used in order to study the effects that a viral leader sequence has on gene expression, then the reporter gene product (GUS, CAT, LUC) is easily screened using its appropriate substrate *(5–8)*; or if a gene encoding for a protein to which an antiserum exists, then this antiserum can be used to detect the expression of that protein

This chapter describes PEG-mediated DNA uptake into tobacco protoplasts and the subsequent detection of coat protein (CP) gene expression by Western blotting

## 2. Materials

### 2.1. Protoplast Isolation and Transformation

1 Mature tobacco plants (mature leaves are easier to peel; *see* **Note 1**)
2. Narrow-tipped tweezers, 64-μm Nitex sieve, parafilm, 9- and 5-cm Petri dishes
3 Hemocytometer
4. Protoplasting solution 0 2 g/L macerozyme (Sigma, Dorset, UK), 1 0 g/L cellulase (Sigma), 80 μg/L mannitol, 20 g/L sucrose, 2 35 g/L MS salts, pH to 5 6 with 0.1*M* NaOH The protoplasting solution was made up in 50-mL amounts, filter-sterilized and stored frozen
5 Protoplast wash solution· 80 g/L mannitol, 2 35 g/L MS salts, pH to 5 6 with 0 1*M* NaOH. The protoplast wash solution was made up in 50-mL amounts, filter-sterilized and stored frozen
6 21% Sucrose solution, filter-sterilized, and stored at room temperature
7 PEG solution 250 g/L PEG 6000, 23 6 g/L $Ca(NO_3)_2$ $4H_2O$, 82 g/L mannitol, 3 9 g/L MES, pH to 6 0 with 0 1*M* NaOH. This solution is made up in a 50-mL amount, filter-sterilized, then aliquoted into 1.5-mL microcentrifuge tubes and stored at –20°C
8 0 275*M* Calcium nitrate solution 65 g/L $Ca(NO_3)_2$ $4H_2O$ and 2 0 g/L MES, pH to 6 0 with 0.1*M* NaOH, and autoclaved
9 Protoplast recovery medium 80 g/L mannitol, 20 g/L sucrose, 2 35 g/L MS salts, pH to 5 6 with 0 1*M* NaOH, filter-sterilized and stored frozen
10 Fluorescein diacetate (FDA) stock solution. 2 mg/mL FDA prepared in acetone and stored in the fridge

### 2.2. Electrophoresis and Western Blotting

The techniques of polyacrylamide gel electrophoresis (PAGE) and Western blotting are described elsewhere in this book.

## 3. Methods

### 3.1. Protoplast Isolation

1 The lower epidermis of a *Nicotiana tabacum* leaf is removed by peeling with a pair of tweezers (*see* **Note 1**)

2 Peeled sections are placed peeled side down into 9-cm Petri dishes containing 30 mL of protoplasting solution Care is taken not to place peeled sections on top of one another (*see* **Note 2**)
3 The Petri dishes are sealed with parafilm to prevent spillage, and incubated overnight on the bench
4 The following morning the dishes are lightly swirled to loosen any protoplasts still attached to the leaves They are allowed to sit for a further half hour before being passed through a 64-μm Nitex sieve (*see* **Note 3**)
5 The protoplasts are gently pipeted into a 50-mL screw-cap tube and collected by centrifugation at 300*g* for 5 min (*see* **Note 4**). The supernatant is removed and the protoplasts are gently resuspended in an equal volume of protoplast wash solution The protoplasts are then collected by centrifugation at 300*g* for 5 min and the protoplasts resuspended in one-third vol (approx 10 mL) of protoplast wash solution
6 2.5 mL of 21% sucrose solution is pipeted into the bottom of 10-mL screw-cap tubes Each tube containing 2 5 mL 21% sucrose solution is gently overlaid with 5 mL of washed protoplasts (*see* **Note 5**) The tubes are spun at 500*g* for 5 min with slow acceleration and deceleration A distinct layer of protoplasts is seen to be floating at the interface of the solutions The protoplasts are then removed from the interface by gentle pipeting, and placed in a new tube.
7 Using a measured volume, the number of protoplasts are then counted using a hemocytometer (number of protoplasts/mL = number in five big squares × 100) The general appearance of the protoplasts can also be determined at this stage (*see* **Note 6**)
8 The protoplasts are spun down at 300*g* for 5 min and resuspended in wash solution to approx $5 \times 10^6$ to $1 \times 10^7$ protoplasts/mL

## 3.2. PEG Transformation of Isolated Protoplasts

1 In 10-mL screw-cap tubes, 0 2-mL PEG solution, 20 μg plasmid DNA (in approx 20–40 μL SDW), and 0 2-mL protoplasts are gently mixed and left for 15–20 min at room temperature (*see* **Note 7**)
2 1 mL 0 275*M* calcium nitrate solution is added dropwise to the mixture, with gentle agitation, followed by another 4 mL of 0 275*M* calcium nitrate solution. The mix is left to stand for 10 min
3 Protoplasts are collected at 300*g* for 5 min, then resuspended in 5 mL recovery medium and gently poured into 5-cm Petri dishes, and incubated for 6 h to overnight on the bench
4 The protoplasts are checked for viability by fluorescein diacetate (FDA) staining FDA, when cleaved by protoplasts or plant cell esterases, releases fluorescein, which is retained only within an intact or viable protoplast Therefore, viable protoplasts can be visualized under UV light (*see* **Note 8**) FDA stock solution is prepared (2 mg/mL acetone) and stored in the fridge. One drop of this FDA stock solution is added to a small sample of the protoplast solution and the protoplasts are examined after 5 min by fluorescence microscopy The percentage of proto-

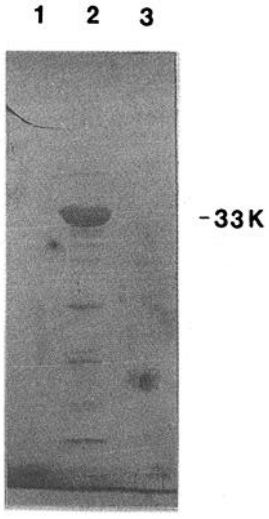



Fig. 1. Western blot analysis of tobacco protoplasts transfected with an expression cassette encoding the PVS 33-kDa CP gene. The PVS CP is detected using commercially available antiserum. Lane 1, protoplasts only (nontransfected); lane 2, protoplasts transfected with recombinant expression cassette, PVS 33-kDa CP detected; lane 3, protoplasts transfected with expression cassette only (not containing the PVS CP gene).

plast viability can be determined by comparing the number of fluorescing protoplasts to the total number of protoplasts.

### 3.3. Detection of CP Expression by Western Blotting

Following PAGE and Western blotting, the protein of interest can be detected using a specific antiserum. The example shown in **Fig. 1** demonstrates the detection of the potato virus S (PVS) 33-kDa CP following its transient gene expression in tobacco protoplasts. The gene encodes the PVS CP and poly(A) sites. Western blots were carried out using specific PVS CP antiserum (many plant virus CP antisera are now commercially available) supplied by Bioreba, Switzerland.

## 4. Notes

1. Peeling of the lower epidermis is less difficult if fine-tipped tweezers are used in conjunction with peeling the epidermis initially from leaf veins, moving out over the leaf surface.
2. It is important not to place peeled sections on top of one another, because this reduces the surface area of cells on which the cell wall degrading enzymes can act.

3 Gentle swirling of the Petri dishes following overnight digestion in protoplasting solution releases the protoplasts from the leaves Sieving of the protoplasts allows separation of the protoplasts from the remaining leaf tissue.
4 During protoplast isolation, gentle pipeting is essential to ensure that protoplasts are not damaged This is achieved by cutting off the ends of pipet tips and slowly resuspending or collecting the protoplasts
5 When the sucrose solution is overlaid with protoplasts, care must be taken not disturb the boundary between the two solutions
6. When looked at under the light microscope, the overall appearance of individual protoplasts is determined If there is evidence of burst cells and "grainy" cellular material among the healthy cells, then it is necessary to carry out a second spin in the sucrose solution, which should remove any debris
7 During PEG transformation of protoplasts with plasmid DNA, the mixture should not be left standing for more than 30 min, because PEG treatment, although less damaging than other such chemicals used, can render protoplasts unrecoverable
8 As experience is gained in isolating protoplasts, the need for FDA staining becomes less essential The general appearance of the protoplasts under the light microscope will allow for determination of viability

## References

1 Dixon, R A (1985) *Plant Cell Culture A Practical Approach* IRL, Oxford, UK
2 Ow, D W, Jacobs, J D, and Howell, S H (1987) Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity *Proc Natl Acad Sci USA* **84,** 4870–4874
3 Guerineau, F., Brooks, L, and Mullineaux, P (1991) Effect of deletions in the cauliflower mosaic virus polyadenylation sequence on the choice of the polyadenylation sites in tobacco protoplasts *Mol Gen Genet* **226,** 141–144
4 Draper, J, Scott, R, Armitage, P, and Walden, R (1988) *Plant Genetic Transformation and Gene Expression A Laboratory Manual* Blackwell Scientific Publications, Oxford, UK.
5 Jefferson, R A., Kavanagh, T A., and Bevan, M. W (1987) GUS fusions. β-glucuronidase as a sensitive and versatile gene marker in higher plants *EMBO J* **6,** 3901–3907
6. Turner, R., Bate, N, Twell, D, and Foster, G. D (1994) Analysis of a translational enhancer upstream from the coat protein open reading frame of potato virus S *Arch Virol* **134,** 321–333
7 Gallagher, S R. (1992) Gus protocols, in *Using the Gus Gene as a Reporter of Gene Expression* Academic, San Diego, CA
8. Allam, J. and Cook, J. L (1990) Reporter genes. application to the study of mammalian gene transcription *Anal Biochem* **188,** 245–254

# 33

# DNA Sequencing

**Jo Badge**

## 1. Introduction

Although the question of whether viruses can be classified in any way into a phylogenetic organization is still hotly debated, the fact that nucleotide sequence data can provide useful information on the grouping together of certain unknown viruses is becoming recognized. It has been shown that, within the *Potyviridae*, which is the largest and most sequenced group of the plant viruses to date, the coat proteins (CPs) of strains of the same virus have an amino acid sequence identity of 90% or more, compared to that of distinct viruses that have an amino acid sequence identity in the range of 38–71% *(1)* Nucleotide sequencing of viral genomes may therefore prove to be a useful tool in their identification

The method of sequencing chosen will depend on the type of virus Viral genomes can be sequenced directly, although many researchers prefer to clone the genome to ensure it is kept in a robust form. Double-stranded DNA viruses can be cloned directly by digestion with appropriate restriction enzymes. However, because the majority of plant viruses are positive-sense single-stranded RNA, another approach is needed. The genome can be reverse-transcribed and the resulting first-strand cDNA made into double-stranded cDNA and cloned directly into a blunt cut vector for sequencing. A library of clones containing viral cDNA inserts can then be sequenced. A miniprep quantity of DNA can be sequenced by double-stranded DNA sequencing. This involves the alkali denaturation of the template, which is then ethanol-precipitated, in order to maintain the denatured state of the DNA The template is then annealed to a primer, which is a short oligonucleotide sequence complementary to the template or to the multiple-cloning site of the vector used. A DNA polymerase (often *Klenow* or T7) is used to extend the complementary strand of the template DNA from

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

the primer, using a mix of dNTPs, which includes radioactive $^{35}S$ αdATP, to label the complementary DNA This reaction is very fast and need only be allowed to continue for a matter of min. The labeled DNA is then transferred to tubes containing one of the four termination mixes. These are mixes of dNTPs containing a specific dideoxynucleotide, which, when incorporated into the complementary DNA strand, will stop the polymerase action by preventing any further nucleotides from binding to it. Termination reactions for each nucleotide A, C, G, and T are then loaded in four adjacent lanes of a denaturing polyacrylamide gel and separated by electrophoresis. Autoradiography is used to detect the separated fragments, so that a permanent record of the sequence can be analyzed.

Single-stranded sequencing uses the same labeling and termination reactions, but no denaturation step is required. Single-stranded DNA (ssDNA) can be made efficiently by using a helper phage. Vectors that contain an origin of replication enable a helper phage to export a single-stranded molecule that will include the insert sequence. This protocol offers a greater degree of reliability and consistency than double-stranded sequencing, and it is much quicker to perform. The least time-consuming method of sequencing, however, is automatic cycle sequencing

Dye-terminated cycle sequencing utilizes the polymerase chain reaction (PCR). Double-stranded or single-stranded template can be used The polymerase used is a thermal-stable *Taq*, and the labeling and termination reactions take place in a thermal cycler, with a fluorescent dye label, not a radioactive one. The resulting fragments are separated by electrophoresis and a chromatogram of the fluorescent dyes present is created and analyzed by computer. The sequence is read automatically and stored as a computer data file.

It is possible to sequence directly the inserts generated for cloning by first-strand cDNA by employing reverse-transcriptase PCR (RT-PCR) Here, first-strand cDNA is created using a primer with a known anchor sequence. This cDNA is then annealed to a second primer and used in a PCR reaction. The first cycle of the PCR will form a double-stranded molecule from the first-strand cDNA, and then PCR will proceed as normal. PCR products can be cloned into vectors specially designed for this purpose (for example, TA pCRII, Invitrogen), or they can be sequenced directly.

In order to sequence PCR products directly, it is necessary to remove all traces of dNTPs, single-stranded template, and primers from the template to be used. This can be done in a variety of ways, by enzymatic treatment, gel, or column purification. If the quantity of PCR product available is high, then it is possible to use manual sequencing techniques to obtain a result. However, automatic sequencing of such products is recommended

## 2. Materials

### 2.1. Double-Stranded Sequencing

1 Sterile $dH_2O$ (SDW)
2 Freshly made 2*M* NaOH
3 3*M* NaOAc
4 100 and 70% ethanol
5 $^{35}S$ αdATP.
6 Either $^{T7}$Sequencing kit (Pharmacia, Uppsala, Sweden) or Sequenase kit (USB, Cleveland, OH)
7 X-ray film

### 2.2. Preparation of Sequencing Gels

#### 2.2.1. TBE Gel

1 A siliconate such as Replicote (BDH, Poole, UK)
2 Industrial methylated spirits or ethanol for cleaning plates
3 Acrylamide mix: 40% 19 1 acrylamide *bis*-acrylamide (*see* **Note 5**)
4 Amberlite (BDH)
5 10X TBE for 1 L 108 g Tris, pH 8 5, with HCl, 55 g boric acid, 9.3 g EDTA
6 Urea
7 10% Ampersulfate
8. TEMED
9. Gel fixer. 10% methanol, 10% acetic acid.
10 Whatman paper.
11. Gel kit (e.g., Bio-Rad Sequi-Gen 21 × 50 cm)

#### 2.2.2. Buffer Gradient Gel

1. 10X Bromophenol blue 0 5 mg/mL
2. Sucrose

### 2.3. Single-Stranded Sequencing

#### 2.3.1 Preparation of ssDNA

1 2X YT broth—per liter to 900 mL deionized water, add 16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g NaCl, shake well, until all solutes have dissolved, and adjust to pH 7 0 with NaOH; make up to 1 L and autoclave
2 Helper phage as appropriate for the vector used, e g., M13K07 for pBSK+
3 Ampicillin: Made at 25 mg/mL, store for 1 mo at –20°C
4 2 5*M* NaCl, 20% PEG 6000.
5. Phenol chloroform
6 10*M* Ammonium acetate
7 Ethanol: 100 and 70%

### 2.4. Automatic Sequencing

#### 2.4.1. Sequenase PCR Product Sequencing Kit

Sequenase PCR product sequencing kit.

#### 2.4.2. Gel Purification of PCR Products

1 Bio 101 gene clean kit
2 Agarose
3 1X TAE: 400 m*M* Tris, 12% acetic acid, 10 m*M* EDTA

## 3. Methods

### 3.1. Double-Stranded Sequencing

Double-stranded sequencing produces reliable sequencing if source DNA is clean (*see* **Note 1**) A cesium-chloride (CsCl) prep is not necessary, in fact, sequencing can be carried out from miniprep quantities of DNA. However, when more than one reaction per sample is required, it is convenient to make a stock of DNA by using an alkali-lysis maxiprep method. This method is outlined using the $^{T7}$Sequencing kit; an alternative kit, Sequenase, by US Biochemicals, has a very similar method; instructions supplied with both kits are easy to follow

1 Use 2–5 µg of DNA and make up to a volume of 32 µL with SDW
2 Add 8 µL of freshly made 2*M* NaOH to denature the DNA Vortex and leave at room temperature for 10 min
3 Add 4 µL of SDW, 7 µL of 3*M* NaOAc, and 120 µL ice-cold ethanol to precipitate the denatured DNA Place at –70°C for 15 min. Microcentrifuge at 12,000*g* for 15 min to pellet the DNA The pellet will be very small and may not even be visible Wash the pellet very gently in 70% ice-cold ethanol and microcentrifuge for 15 min at 12,000*g*
4 Dry the pellet under vacuum and resuspend in 10 µL SDW (*see* **Note 2**)
5 Add 2 µL of primer and 2 µL of annealing buffer and incubate at 65°C for 5 min Transfer to a 37°C water bath for 10 min Place at room temperature for 5 min During this time, label four tubes for each reaction A, C, G, and T In each tube, place 2.5 µL of the appropriate termination mixture (A, C, G, or T)
6 Once the incubation period is over, add 3 µL labeling buffer, 0 5 µL of $^{35}$S αdATP, and 2 µL of diluted T7 enzyme to the DNA and primer mixture (*see* **Note 3** for different method for long runs).
7 Incubate the labeling mix at room temperature for 5 min. The termination reactions must be placed at 37°C for at least 1 min before the labeling mixture is added
8 Transfer 4 5 µL of labeling mixture to each of the four termination reaction tubes A, C, G, and T Incubate for 5 min at 37°C Add 5 µL of stop mixture and mix by pipeting The reactions are now ready for loading onto a gel They can be stored temporarily at –20°C or long-term at –70°C

### 3.2. Preparation Sequencing Gels

Gel kits will vary in size and shape, but this general method can be adapted. A TBE gel is suitable for short runs (up to 400 bp from the primer site) and long runs, though it may be necessary to change the running buffer on runs of over 4 h, because it may become exhausted. To increase the number of bands that can be read from one gel, particularly on a long run, a buffer gradient gel can be used. For very long runs, it may be necessary to use a different percentage of acrylamide (*see* **Note 4**) to improve resolution. Many ready-prepared commercial gel mixes are now available and reduce the risks involved with handling powdered acrylamide. The mixes of 40% 19·1 acrylamide not only have a longer shelf life than premade mixes, but are also more versatile, allowing you to customize your gels, as needed, to maximize the amount of sequence read from one gel (*see* **Note 5** for making 40% acrylamide).

#### 3.2.1. TBE Gel Preparation

This method applies to the BioRad Sequi-Gen kit 21 × 50 cm gel, but is similar to that required for other kits and can be easily adapted.

1. Prepare the gel kit Ensure that clips fit well and make a tight seal Clean the surfaces of the glass plates that will be in contact with the gel, first with water and detergent, then rinse and wash with ethanol or industrial methylated spirits Dry and coat the top plate with siliconate to facilitate its removal once the gel has run Allow to dry in a fume hood
2. Prepare the gel mix This will vary depending on the percentage of gel required (*see* **Note 4**), for most short runs, a 6% acrylamide gel is used For 100 mL (enough for one gel), mix 15 mL of the ready-prepared 40% acrylamide mix with 50 g of urea (*see* **Note 5**) The large amount of urea will be difficult to dissolve, especially because this is an endothermic reaction To facilitate this process, warm the mixture on a heated stirrer Finally, add 10 mL of 10X TBE and 35 mL of distilled water to make the volume up to 100 mL.
3. Assemble the kit, clipping both plates together, with spacers inserted, ensuring that the bottom of the spacers and the two glass plates are flush with the bottom of the clips
4. Line the casting tray with a strip of Whatman paper To 40 mL of gel mix, add 480 µL of 10% AMPS and 64 µL TEMED, mix well, and pour into casting tray Push the bottom of the gel plates into the tray so that the acrylamide is forced up between the plates Secure the position of the plates by tightening the screws on the tray. Leave to stand in a vertical position for 10 min to allow the plug to set
5. Pour enough water down the back plate into the buffer reservoir to cover the electrode This will prevent any acrylamide setting round the electrode, if it overflows into the reservoir tank by mistake.
6. To 60 mL of gel mix, add 480 µL of 10% AMPS and 64 µL TEMED, mix well. Using a 100-mL clean syringe, suck up the acrylamide mixture and pour

between the plates Pour smoothly and continuously down the far side of the gel, holding the plates at an angle away from your body Then tilt the gel, still pouring continuously, toward your body to allow the acrylamide to cover the bottom of the gel Continue to pour until the gel is almost complete, lower the top end of the gel, when the acrylamide is a few centimeters from the top of the plates, onto an empty tip box, or some other stand This will allow the acrylamide to settle into position If any bubbles form in the gel, raise the plates to a more vertical position to force them to rise They can then be reached by a bubble getter (these are available commercially, but a piece of developed X-ray film cut into an extended hook will work just as well) and hooked out of the gel Push in the shark-tooth comb, with the flat edge toward the bottom of the gel, to make an insert for the teeth of the comb when it is set.

7 Clamp the comb to ensure a close fit and leave the gel to set in this propped-up position for 1 h. The gel can be left overnight and run within 24 h, if the top of the gel is covered with cling film or a layer of 1X TBE running buffer, as oxygen will inhibit polymerization of the gel

8 Once set, remove the plug and casting tray and the clamps, leaving the comb in place Make up 1 L of 1X TBE running buffer Place the gel in the running tank and secure in place Pour the water out of the buffer reservoir at the back of the plates and fill it with running buffer. Pour the rest of the buffer into the bottom reservoir to cover the electrodes

9 Remove the comb carefully, using forceps, pulling it out evenly, to prevent it from slipping on an angle and denting the top of the gel

10 Using a syringe with a fine needle, wash out the space left by the comb to remove any crystals of urea or pieces of loose acrylamide Wash the comb and replace it, teeth down, into the space at the top of gel, taking care not to push it into the gel, but leave it just touching on the top edge Using the syringe, wash out each well carefully, and check that the seal on each is tight by looking for buffer leaking between wells as you wash

11 Attach the electrodes and prerun the gel at 55 W or 2500 V until the temperature indicator on the gel registers 50–55°C Alternatively, prewarm the running buffer to 50°C in a microwave before pouring into the gel apparatus; this will cut down the time needed for the gel to reach the set temperature

### *3.2.2. Preparation of a Buffer Gradient TBE Gel*

This gel has a graded concentration of salt, which increases toward the bottom of the gel. This has the effect of creating a voltage gradient down the length of the gel, which slows the migration of low mol-wt fragments near the bottom of the gel. The bands are more evenly spaced on the final autoradiogram, and thus more sequence can be read from one run (up to 600 nucleotides), as compression at the top of the gel is reduced, and resolution is improved

1 Prepare the plates as for **Subheading 3.2.1.**
2. Mix two gel solutions
   a. 75 mL Light solution (0 5X TBE, 6% acrylamide, 7 67*M* urea)
   b. 40 mL Heavy solution (2 5X TBE, 6% acrylamide, 7 67*M* urea, 10% sucrose, 1X bromophenol blue)
3 Pour the light and heavy solutions into separate beakers.
4 Add 10% AMPS and TEMED to achieve a final concentration of 0.25% AMPS and 0.1% TEMED to 20 mL of the heavy solution to use for a casting plug, as described in **Subheading 3.2.1.** Once set, add AMPS and TEMED to both remaining solutions
5. Take up 50 mL of the light solution into a 50-mL syringe and set aside
6 Take up 12 mL of light solution into a glass 25-mL pipet Then take up 12 mL of the heavy solution into the same pipet This should mix slightly on the interface to form a gradient; if it does not, allow a couple of air bubbles to be drawn into the pipet
7 Pour this gradient down the center of the gel
8 Pour the rest of the light solution into the gel, making sure that the flow is kept in the center of the gel, so that the gradient forms evenly across the gel, as well as vertically along its length The bromophenol blue dye will show how well the gradient has formed
9 The gel can be run in the usual way The bromophenol blue dye will run out of the gel as electrophoresis proceeds, so the progress of the fragment dye front can be seen The dark-blue dye front should take at least 4 h to run off the gel

### 3.2.3. Loading, Running, and Drying Sequence Gel

1 It is important to clean the wells created by the shark-tooth comb before every loading of the samples, particularly if the gel is reloaded after a period of time (*see* **Note 6**).
2 Place the sequencing reactions in a 80°C water bath for 2 min. Place immediately on ice and load 2–3 µL of each reaction on the gel The loading order is a matter of personal choice, though for a long run a special method of loading, which places every base next to every other base, is recommended to enable more sequence to be read (*see* **Note 6**).
3. Run the gel at 2500 V or 50 W until the dark blue dye has reached the bottom of the gel for a short run (approx 2 h). Running a gel on constant power (watts = volts × amps) prevents large surges of voltage or overheating occurring, the amount of heat generated will affect the resistance of the gel and, therefore, the current through it
4 Short runs to cover distances up to 250 bp from the primer usually take 2–3 h, depending on the type of gel used A good indicator of how far the gel has run can be gained from the position of the two dye fronts (*see* **Note 7**) Once the gel had finished running, disconnect it from the running apparatus and lay it down, resting on the back plate Remove the clips carefully and prize off the top plate using first finger and thumb at the top corner of the plates. Carefully lift off the upper plate, leaving the gel on the back plate.

5 The gel can be transferred onto Whatman paper at this point and dried under vacuum at 80°C, with a trap attached for 2 h, or it can be fixed first Fixing the gel should reduce its drying time to 30-45 min by removing the hygroscopic urea in the gel. Place the gel and back plate in a large tray, pour the fix over the gel, and make sure the gel is covered for 15–20 min Carefully pour off the fix, and transfer to Whatman paper and dry in the usual way
6 Expose the dried gel to X-ray film overnight Develop and dry the autorad to read the sequence (*see* **Notes 8–10** for problem-shooting on the appearance of the sequence)

## 3.3. Single-Stranded DNA Sequencing

### 3.3.1. Preparation of Single-Stranded DNA

pGEM-T (Promega) and Bluescript (Stratagene) contain F1 origins of replication enabling the export of a single-stranded molecule, including insert sequences. Single-stranded sequencing offers a speedier protocol, longer reads (up to 700 bp), and better clarity and resolution than double-stranded sequencing.

1 Add the following to a 50 mL sterile screw-top plastic tube 5 mL LB broth, 25 µL M13KO7; 24 µL Ampicillin (25 mg/mL), 100 µL fresh overnight culture of pGEM-T or Bluescript clone
2 Incubate at 37°C overnight in a shaking incubator.
3 Spin the whole 5-mL culture at 12,000*g* in a benchtop centrifuge for 5 min to pellet the cells
4 Split the supernatant into three microcentrifuge tubes, placing 1 5 mL in each, and microcentrifuge for 5 min at 12,000*g*
5 Remove 1 mL of supernatant from each of the tubes to fresh Eppendorfs containing 200 mL of 20% PEG 6000/2 5*M* NaCl, and mix by inverting
6. Leave at room temperature for 20 min
7 Microcentrifuge for 10 min at 12,000*g* and pour off most of the supernatant
8 Recentrifuge for a further 3 min and remove the remaining PEG mixture with a drawn-out Pasteur pipet The white pellet of phage should be visible at this stage
9 Add 100 mL sterile distilled water to each pellet, resuspend, and add 50 mL of phenol. Vortex for 10 s
10 Microcentrifuge for 3 min at 12,000*g* and transfer upper aqueous layer to a fresh Eppendorf, bulking up the contents of the three tubes into one
11. Extract once more with an equal volume of chloroform
12 Add 0.25 vol of 10*M* ammonium acetate and 2 vol of absolute ethanol Mix and store at –20°C until required
13. Precipitate the ssDNA by centrifugation for 10 min at 12,000*g* Wash the pellet in 1 mL 70% ethanol and vacuum dry
14 Resuspend the pellet in 40 µL of water Check the quality of the ssDNA by spectrophotometer analysis or on a normal 1% agarose gel (*see* **Note 11**)

### 3.3.2. Labeling and Termination Reactions for ssDNA

Because the single-stranded DNA needs no prior treatment, the labeling reactions can be started immediately. The same method as described for double-stranded sequencing is followed, except that annealing of the primer to the template is performed at 60°C for 10 min, instead of the quick annealing and slow cooling of the dsDNA method (*see* **Subheading 3.1., steps 5–8**).

## 3.4. Automatic Sequencing

These notes are based on those supplied with the ABI PRISM (Perkin-Elmer) dye terminator cycle sequencing ready reaction kit. Information is also available from the Perkin-Elmer user bulletins on DNA preparation, and other protocols for template production.

### 3.4.1. Preparation of DNA for Automatic Reactions

A small amount of DNA is required for automatic sequencing, but it must be extremely clean The best method for easy and consistent preparation of dsDNA for automatic sequencing is one that involves a column purification step without the use of PEG, or phenol:chloroform. There are many such kits available; one example is Qiagen DNA isolation system, which uses a 5-mL overnight culture. Single-stranded DNA can also be used in these reactions and can be prepared in the same way as previously described Symmetric PCR templates can be used, and, although these are usually difficult to denature, cycle sequencing can overcome this problem.

### 3.4.2. Direct Automatic Sequencing of PCR Products

Specifically amplified PCR products can be utilized directly in sequencing reactions if components of the PCR reaction that interfere with sequencing are removed, either by enzymatic degradation or by gel purification. Direct sequencing can be used as a rapid and representative way to sequence PCR products The following protocol is that recommended by the manufacturers of the Sequenase PCR Product Sequencing Kit

#### 3 4 2 1. Enzymatic Treatment

1. Examine yield and specificity of PCR by agarose gel electrophoresis If a single specific product is present, this may be used directly in sequencing reactions
2. To 5–7 µL of PCR reaction mix, add 1 µL *Exonuclease* I (10 U/µL) and 1 µL shrimp alkaline phosphatase (2 U/µL). Mix and incubate at 37°C for 15 min.
3. Inactivate *Exo*I and SAP by heating to 80°C for 15 min Both incubation steps can be carried out in a thermal cycler with an appropriate program.
4. To the treated PCR product, add 1 mL of primer solution (10 pm/mL) and SDW to 10 µL

5 Heat to 100°C for 3 min in a thermal cycler and then plunge into an ice-water bath Centrifuge briefly to collect contents, then store annealed template on ice until required (use within 4 h)
6. The product can be sequenced using the same labeling and termination reactions described in **Subheading 3.1., steps 5–8** or used as the template for automatic sequencing (*see* **Note 12**)

### 3.4 2 2 Gel Purification

1. Examine yield and specificity of PCR product by agarose gel electrophoresis The whole PCR sample can be loaded directly onto the gel
2 Use a scalpel blade or razor blade to excise the band required in the gel and place in a clean Eppendorf tube
3 Follow the instructions supplied by the manufacturers with the Bio-101 GeneClean kit to extract the DNA sample from the agarose gel
4 Determine the concentration of the purified DNA by agarose gel electrophoresis. Use the required amount of DNA in the automatic sequence reactions (*see* **Subheading 3.1.**)

## 4. Notes

1 For successful double-stranded sequencing, DNA should be clean and concentrated to approx 1 µg/mL. Check that the sample is free from RNA by electrophoresing on an agarose gel The sample should give a smooth curve when analyzed by spectrophotometry on a spectrum between 200 and 300 nm, a convex slope in the 200 to 250-nm wavelength range indicates too much salt in the sample (to remove this, wash with 70% ethanol and dry thoroughly under vacuum); a rise at the very end of the profile indicates trace phenol. chloroform (repeat the phenol chloroform, taking care not to carry over any into the aqueous layer)
2 It is best to carry on immediately with the labeling reactions, once the denaturing step has been completed
3 For long reactions (to read more than 400–500 bp from the primer), a slightly different labeling reaction can be used to make sure that the polymerase extends to the region of sequence needed. Although the T7Sequencing and Sequenase kits employ different strategies for long reactions, there are two points common to both. first, that the incubation time of the labeling reaction should be increased from 5 min to 10 or even 15 min Second, that the amount of $^{35}$S αdATP used in the reaction should be increased to 1 µL

The T7Sequencing kit comes with two different types of termination reactions: read short and read long. These contain different molar amounts of dideoxynucleotides to alter the frequency of their incorporation Use the read-long mixes in the termination reactions. The Sequenase kit has only one set of termination reactions, but here the labeling buffer is usually diluted to 1 × for short runs. For a long run, use the labeling buffer neat

4 A 6% acrylamide gel should produce 300–400 nucleotides of clear sequence To visualize sequences close to the primer (within 50 nucleotides), use a higher-percentage gel (12–20%), to read more distal sequences, use a lower percentage gel (4%) Take care when running these gels, because high-percentage ones may have a tendency to overheat as a result of their increased resistance, and, conversely, lower-percentage gels may melt while running, and are less robust on removal of the top plate and subsequent transfer to paper For a more uniform spacing of gel bands, use wedge-shaped spacers or a gradient buffered gel (*see* **Subheading 3.2.2.**)

5 40% 19 1 acrylamide *bis*-acrylamide This should be prepared in advance and can be stored indefinitely at 4°C, if protected from the light **Note:** Take great care when handling acrylamide It is a neurotoxin Always wear gloves, and weigh out and dissolve in a fume hood Heat will be needed to dissolve the urea Deionize for 1 h using amberlite, filter through Whatman paper to remove any metal ion impurities that may be present.

6 When loading gels, it is important to wash the wells with buffer before every loading, especially when loading onto a gel that has already been running for several hours This removes any crystals of urea that form as the gel heats up and cause the bands to look very jagged, which can make them difficult to resolve The wells can be rinsed easily with a syringe and narrow gage needle, using the buffer in the backplate reservoir

7 The migration of loading dyes in TBE denaturing polyacrylamide gels

| Gel % | Comigrating DNA fragment size | |
|---|---|---|
| | Bromophenol blue | Xylene cyanol |
| 6% | 26 bp | 106 bp |
| 8% | 19 bp | 75 bp |
| 10 | 12 bp | 55 bp |

8 To read sequences close to the primer use a manganese buffer supplied with the Sequenase kit

9 Band compressions often occur in G–C-rich regions of sequence and they can mask the correct sequence in a particular region of the gel Compressions are fragments that have comigrated because they are not properly denatured as a result of their formation of stable intrastrand secondary structures This problem can sometimes be overcome by repeating the sequencing reactions using different termination mixes that contain 7-deza dGTP, 7-deza dATP, or ITP, which are purine analogs

10 Parallel bands in all four lanes at sequences 200 bp or more from the primer can be a common problem It may be caused by a decrease in enzyme activity, if the termination or labeling reactions were carried out at temperatures above 37°C Take care to use a properly equilibrated water bath or incubator for these stages Another possibility is that the enzyme may have lost its activity by repeated freeze–thaw cycles Increase twofold the amount of enzyme used in each reaction The template may also have strong secondary structures that cause the polymerase to pause Following the annealing step, incubate the primer–template

solution at 60–70°C for 4 min, return the solution to room temperature for 2 min and then immediately proceed to the labeling reaction

11. Single-stranded DNA quality can be assessed by the $A_{260}/A_{280}$ ratio, which should not be greater than 1 8 for successful sequencing If the absorbance is greater than 1.8, then repeat phenol chloroform extractions If the preparation is clean, then the $A_{260}$ can be a good estimate of the concentration of the ssDNA A 1 mg/mL solution of ssDNA has an $A_{260}$ of 33 Quality and quantity of ssDNA can also be determined by agarose gel electrophoresis, using a 1% EtBr stained gel Two major bands are usually seen the helper phage DNA and the single-stranded plasmid There may be genomic chromosomal contamination, which will appear as a large product, or RNA contamination, which will appear on the gel as a smear of small fragments
12. With the Sequenase PCR product kit, the polymerase is supplied prediluted in a modified buffer This contains inorganic pyrophosphatase to prevent pyrophosphate accumulation and also a higher than normal glycerol concentration to aid enzyme stability For this latter reason, direct sequencing reactions must be run on glycerol-tolerant sequencing gels (i e , not TBE gels) This preparation is compatible with the use of 7-deaza-dGTP to resolve compressions and manganese buffer to read close to the primer

## Reference

1 Shukla, D D and Ward, C W (1988) Amino acid sequence homology of coat protein as a basis for identification and classification of the potyvirus group *J Gen Virol* **69,** 2703–2710

# 35

# Preparation of Coat Protein-Containing Binary Vectors for Use in Agrobacterium-Mediated Transformation

**Corrin V. Wallis and Margaret I. Boulton**

## 1. Introduction

Transgenic plants expressing the coat protein (CP), and/or the RNA transcript of the CP gene of many RNA viruses, may be protected against the virus from which the CP gene was derived, and, in some cases, against related viruses (for further details, *see* Chapter 3). Since it is not possible to predict from the literature when protein expression is not required, the procedure described in this chapter will be designed to obtain CP expression. The initiation codon of the CP may be deleted or mutated to prevent production of the protein

To date, the majority of CP-mediated resistant transgenic plants have been dicots, since the majority of species are more amenable to transformation by the naturally occurring soil bacterium *Agrobacterium tumefaciens* and are more easily regenerated to whole plants than monocots However, recent improved regeneration efficiency of cereals (especially rice and maize) and the demonstration that rice may be transformed by *Agrobacterium* ***(1)*** are likely to lead to the extension of the technique to the economically important cereal crops.

Wild-type *Agrobacterium* strains produce tumors on host-plant tissues, which prevent subsequent regeneration of plants; therefore, disarmed strains, in which a portion of the Ti (tumor-inducing) plasmid has been deleted, have been produced, e.g., LBA4404, pGV3850 ***(2,3)***. These strains are nononcogenic, but still possess the virulence (*vir*) genes necessary for the transfer of foreign genetic material to the plant.

For *Agrobacterium*-mediated gene transfer, the foreign DNA must be present between the left (LB) and right (RB) borders of the Ti plasmid. The large size of the Ti plasmid (wild-type plasmids are approx 200 kb) precludes its direct

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

manipulation, and therefore an alternative small plasmid (a 10- to 20-kb binary vector) is used that is able to replicate in *Escherichia coli* as well as *Agrobacterium*. Thus, cloning is done in *E coli* and the final expression cassette in the binary vector is then introduced into *Agrobacterium* containing the *vir* plasmid, using either conjugation (with the help of plasmid pRK2013, which provides the transfer functions for mobilization of plasmids from *E coli* to *Agrobacterium* ***[4]***) or transformation procedures. In the latter case, electroporation of transformation-competent *Agrobacterium* ***(5)*** is the method of choice, although, in the absence of the appropriate equipment, the less-efficient freeze–thaw method ***(6)*** may be used.

The binary vector represents an artificial T-DNA generally defined by the T-DNA borders (although only the RB is essential for transfer, and binary vectors containing only the RB are transfer competent). Inserted between the borders, there are a selectable marker gene expression cassette for selection of transformed plant cells and a site for cloning of the foreign gene. The latter may either constitute a multiple cloning site (mcs) suitable for direct cloning of an already-constructed foreign gene plant expression cassette or an expression cassette composed of a plant transcriptional promoter, a cloning site(s), and a termination (or polyadenylation) signal. The strong constitutive cauliflower mosaic virus (CaMV) 35S promoter and the CaMV or nopaline synthase (*nos*) poly(A) sequence regions are commonly used for transcription initiation and termination, respectively. Presently, there is no evidence to support the requirement for tissue-specific promoters for CP-mediated protection, even for phloem-limited viruses. Thus, the CaMV 35S promoter is suitable for expression in all dicot plants, but alternative promoters may be required for high levels of protein expression in some monocots ***(7)***.

The neomycin phosphotransferase II gene (*nptII*) is a commonly used selectable marker gene that confers resistance to kanamycin in transformed plants. This has proven useful for solanaceous plants, but less so for the more recalcitrant legumes and monocots, for which hygromycin or the herbicide bialophos are commonly used; in these cases, alternative vectors will be required (e.g., *see* **refs.** ***1*** and ***8***).

Binary vectors also possess an antibiotic resistance gene for selection of recombinant bacteria. The presence of the *lacZ* gene may facilitate identification of recombinant clones through blue-white color selection (*see* Chapter 27), but is available on only a few binary vectors (e.g., pBIN19; **ref.** ***9***), and generally not on those that already contain the expression cassette A reporter gene cassette (commonly encoding β-glucuronidase [GUS]) for identification of transformed plant tissues may also be useful. Few of the vectors used for CP- mediated protection contain the GUS gene, although one, pGA482GG

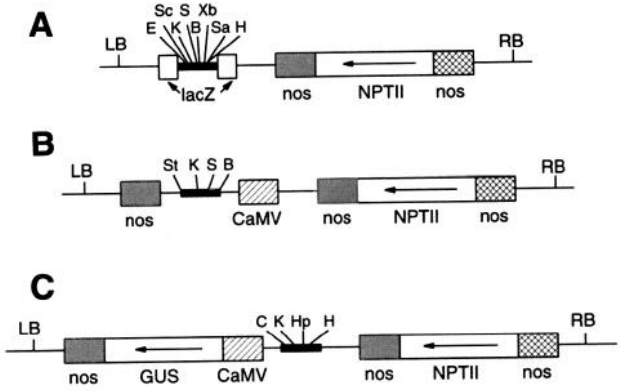



Fig. 1. Examples of types of binary vectors used for *Agrobacterium*-mediated transformation of plant cells. The *npt*II gene provides kanamycin selection for transformed plants, and unique restriction enzyme sites within the multiple cloning sites (mcs, solid bars) facilitate CP gene insertion (only unique sites are shown on this figure). **(A)** pBIN19 ***(9)***, which requires insertion of a CP gene cassette comprising promoter and terminator sequences, but provides color selection from the *lacZ* gene. **(B)** pROKII, which provides transcription regulatory signals flanking the mcs. **(C)** pGA482GG ***(10)***, which includes a GUS marker-gene cassette, but requires a CP gene cassette to be inserted into the mcs. Hatched- and line-filled boxes represent promoters (nos or CaMV, respectively), dot infils the nos terminator. Restriction sites: B, *Bam*HI; S, *Sma*I; K, *Kpn*I; Sc, *Sac*I; H, *Hin*dIII; Hp, *Hpa*I; C, *Cla*I; E, *Eco*RI; Xb, *Xba*I; Sa, *Sal*I.

***(10)***, has proved successful in a number of studies ***(10–12)***. Typical binary vectors are shown in **Fig. 1**.

The artificial T-DNA is transferred to the plant by cocultivation of wounded plant material (generally, leaf disks, *see* Chapter 38) and *Agrobacterium*. The transfer is mediated via the *vir* functions present in *trans* on the Ti plasmid (for a review of *Agrobacterium*-mediated plant transformation, *see* **ref. *13***).

The procedures required to produce CP-expression constructs for *Agrobacterium*-mediated plant transformation can be divided into three steps:

1. Ligation of the virus gene fragment into a plant expression cassette already present in a binary vector, and transformation of *E. coli* with the binary construct.
2. Transfer of the binary construct to *Agrobacterium* by electroporation (method 1) or conjugation (the triparental mating procedure), method 2.
3. Confirmation of the integrity of sequences inserted in *Agrobacterium* by Southern analyses (method 1) and/or the polymerase chain reaction (PCR, method 2).

Cucumber mosaic virus (CMV)-O strain will be used to illustrate the cloning of a plant virus CP gene into the plant binary expression vector pROKII, as

described by Yie et al. *(14)*. Vector pROKII is based on pBIN19, but contains the CaMV 35S promoter and nos termination sequence. For plasmid transfer to *Agrobacterium*, both the triparental mating procedure (modified from **ref. *15***) and the electroporation procedure (modified from **ref. *5***) will be described, because electroporation equipment may not be available in all laboratories.

## 2. Materials

1. A DNA sample of a cloned cDNA copy of the virus CP gene (e g , CMV strain O, clone pUCP)
2. DNA of the *Agrobacterium* binary vector (here pROKII; *see also* **Note 1**).
3. Molecular biology reagents for the purification and cloning of DNA (*see* **Note 2**)
4. The appropriate *Agrobacterium* strain (here LBA4404, but *see* **Note 3**)
5. For triparental mating (**Subheading 3.2.2.**) a helper plasmid (pRK 2013) present in *E coli* (normally HB101) for mobilization of the binary plasmid from *E coli* to *Agrobacterium*, and nitrocellulose filter circles (2 5 cm diameter, 45 µ pore size). Neither of these materials are necessary if *Agrobacterium* is to be transformed by electroporation
5. For electroporation (**Subheading 3.2.1.**). modified YEP broth and agar. Prepare by adding 10 g yeast extract, 10 g peptone, 5 g NaCl, and 5 g sucrose to 950 mL distilled water. Bring to pH 7 5 and make to 1 L. Sterilize by autoclaving
6. Sterilized LB broth and LB agar (*see* Chapter 27)
7. Appropriate filter-sterilized antibiotic solutions (here kanamycin sulfate), 50 mg/mL in $dH_2O$, and rifampicin (50 mg/mL in methanol) (*see* **Note 4**)
8. Sterilized glycerol

## 3. Methods

### 3.1. Cloning of DNA Fragments Into Plant Transformation Vectors

Throughout this chapter pROKII will be used as an example of a binary vector and pUCP *(14)* will be used to provide the virus CP insert. The CP gene may be obtained by digestion of pUCP with *Bam*HI and *Sac*I and inserted into similarly digested pROKII. A non–insert-containing clone should also be produced to provide control transgenic plant lines

1. Digest approx 10 µg of the cloned virus DNA with the appropriate restriction enzyme to release the CP gene fragment from the cloning vector For pUCP, this could be *Bam*HI and *Sac*I (*see* **Note 5**)
2. Separate the CP fragment from the vector by agarose gel electrophoresis The fragment may be visualized by ethidium bromide staining and UV irradiation and purified from the agarose using your preferred technique (*see* **Note 6**)
3. Digest approx 5 µg of the binary vector with the appropriate restriction enzymes (for pROKII, these are *Bam*HI and *Sac*I) and purify the linearized vector from an agarose gel (*see* **Notes 1** and **7**)

4 Ligate the binary vector and CP fragments using T4 DNA ligase and approx 100 ng of the binary vector and a 3–4*M* excess of the CP fragment (in most cases, this will be an approx 20-ng CP fragment; *see* **Note 8**)
5 Transform competent *E coli* cells with the ligation mixes Include a transformation control using approx 20 ng of uncut binary vector Plate the cells onto LB agar containing the appropriate antibiotic (for pROKII, 50 μg/mL kanamycin, *see* **Note 9**)
6 Confirm the presence of the insert by restriction enzyme digestion and agarose gel electrophoresis of small-scale plasmid DNA preparations (*see* **Note 10**)

## 3.2. Introduction of Plasmids into Agrobacterium

### 3 2 1. Using Electroporation

1 Grow an overnight culture (10 mL) of the *Agrobacterium* strain, shaking at 28°C (*see* **Note 11**).
2 For each DNA construct to be used, pellet the bacteria from 2 × 1 5-mL aliquots of the culture by centrifugation for 1 min in a microcentrifuge
3. Wash pellets by resuspension in ice-cold 1 m*M* HEPES/KOH, pH 7 0, followed by centrifugation for 30 s at high speed in the cold
4. Repeat the washing step twice more
5 Resuspend the pellets in 0 5 mL ice-cold 10% glycerol, centrifuge as before
6. Resuspend in 20 μL ice-cold 10% glycerol. Combine the contents of the two tubes
7 Add binary construct DNA (*see* **Note 12**) and leave the tube on ice for 5 min
8. Transfer the bacterial mix to an ice-cold electroporation cuvet (Bio-Rad, 0 2 cm electrode gap) Pulse using a Bio-Rad gene pulser with pulse controller (*see* **Note 13**)
9 Dilute with 1 mL YEP medium, incubate shaking for 2 h at 28°C, prior to plating serial dilutions on selective LB agar (for pROKII, 50 μg/mL kanamycin) Incubate at 28°C for 36–48 h

### 3.2.2. Using Triparental Mating

1 Grow 10-mL cultures of each of the *E coli* donor (containing the binary vector construct), and helper strains (HB101 containing plasmid pRK2013), and the appropriate *Agrobacterium* strain to exponential phase (*see* **Notes 11** and **14**) Include also the binary vector culture (the minus insert control).
2. Centrifuge the bacteria at 3000*g* for 10 min; resuspend in 10 mL LB broth (*see* **Note 15**)
3 Mix the three cultures in the ratio of 1 vol of each *E coli* culture to 2 vol of *Agrobacterium* (e g , 200 200.400 μL, respectively) in a sterile microcentrifuge tube
4 Pipet 400 μL of the conjugation mix onto a sterile nitrocellulose circle positioned in the center of a Petri dish containing LB agar. Allow the filters to dry for approx 1 5 h in a laminar flow cabinet Incubate the dishes and filters at 28°C for 24–36 h *(see* **Note 16**).
5. Place filters in 10 mL of sterile distilled water and shake to resuspend the bacterial cells (*see* **Note 17**)

6 Spread 100 µL of the resulting suspension at dilutions of $10^{-2}$, $10^{-4}$, and $10^{-6}$ on LB agar containing the appropriate antibiotics (e.g , for LBA4404 and pROKII, 50 µg/mL rifampicin plus 50 µg/mL kanamycin, *see* **Note 18**) Aliquots of each of the three bacterial cultures should also be plated Incubate for 48 h at 28°C, to allow single colonies of transconjugants to grow
7 Confirm the fidelity of the *Agrobacterium* transconjugant using either Southern hybridization or PCR analyses, and store the bacteria in 50% glycerol at –70°C (*see* **Note 19**)

## 3.3. Analysis of Transconjugants

### 3 3 1. Using Miniprep and Southern Analyses

1. Prepare the binary vector plasmid DNA using standard miniprep protocols for *E coli*, except that *Agrobacterium* should be grown for approx 24 h at 28°C (*see* **Note 20**).
2 Digest approx 5 µg of the DNA with restriction enzymes that produce a restriction fragment pattern characteristic of the binary vector construction (*see* **Note 21**)
3 Separate the DNA fragments by agarose gel electrophoresis, analyze using Southern blotting and hybridization to a labeled CP-specific DNA probe (*see* **Note 22**) The techniques are detailed in Chapters 27, 41, and 43.

### 3.3.2. Using PCR Techniques

The amplification of the CMV-O CP insert using primers that bind to the CaMV 35S promoter and the nos terminator will be described (*see* **Note 23**).

1 Use 1 µL (or 2% of the total volume) of miniprep DNA (e g , that prepared in **Subheading 3.3.1., step 1**) or a small proportion of the *Agrobacterium* colony under test
2 Carry out a PCR reaction as described in Chapters 28 and 42, using the following primers
   a. Primer 1 (CaMV 35S promoter). 5'-ATATCTCCACTGACGTAAGG-3'
   b Primer 2 (nos terminator) 5'-CGGCAACAGGATTCAATCTT-3'
   and reaction conditions of: Denaturation at 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 53°C for 2 min, and 73°C for 3 min (*see* **Note 24**)
3 Confirm the presence of the insert within the transconjugant *Agrobacterium* by electrophoresis of 10% of the amplified sample (*see* **Note 24**)

## 4. Notes

1 Many binary plant transformation vectors are freely available for research, however, for work that is likely to have commercial applications, the use of some vectors produced with industrial funding may be restricted The vector (pROKII) that we have chosen as an example is freely available and does not suffer from such limitations Although it has been found that pBIN19 and its derivatives (e g , pROKII) have a mutation within the *nptII* gene that provides

transgenic plants with suboptimal resistance to kanamycin, the vectors have been used frequently to produce CP-mediated virus resistance and are not likely to present problems with any commonly used plant species. The complete sequence of pBIN19 is known ***(16)***, and an improved version, pBINPLUS has been produced ***(17)*** The choice of binary vector may also depend upon the following.

a The suitability of restriction enzyme sites (e g , a multiple cloning site) for the CP gene sequence

b The compatibility of the bacterial selectable marker gene with the *Agrobacterium* strain to be used for plant transformation (many disarmed strains carry some bacterial resistance, e.g., pGV3850 is carbenicillin- and rifampicin-resistant and therefore not suitable for use with binary vectors that are selected for by carbenicillin resistance)

c The ability of the binary vector to be maintained in the absence of selection The latter is of relevance when assessing risk factors, because a low-copy-number vector, which is rapidly lost without selection, has a lower environmental risk than one that is more stable.

Many of the modern binary vectors are more practical, having a wider range of unique enzyme sites to facilitate cloning, either through inclusion of rare restriction enzyme sites and/or decreased vector sequences A number of versatile vectors have been constructed by Jones et al ***(18)*** that contain different promoters, terminators, and selection cassettes, as well as an array of restriction sites, to facilitate direct insertion of foreign genes or the transfer of foreign-gene cassettes from compatible pUC-based plasmids Additionally, they allow a choice of orientation of the expression cassette that may be of importance for optimal expression of transgenes (*see* references within **ref. *18***) Although the vectors have not been used for CP gene insertion, there is no evidence to suggest that choice of binary vector is important for CP-mediated resistance Applications for vectors should be directed to the laboratories in which they were developed.

2 Molecular biology protocols used here are described in Chapters 27, 28, and 41–43 Additional information may be obtained from **ref. *19***

3 The choice of *Agrobacterium* strain depends on its host range (i e , the ability of the bacterium to interact with the host plant). As well as the strains described in **Subheading 1**, wide host-range disarmed supervirulent strains, such as AGL1 ***(20)*** and EHA105 ***(21)***, are available. A survey of the literature should show strains suitable for transformation of particular plant species (for example, *see* references within Table 3 2 of Grumet ***[22]***). The correct choice of strain is especially important if cereals or grasses are to be transformed ***(1,23)***

4. Some antibiotics are degraded by light, but most solutions are stable if stored at –20°C All should be handled with care (use gloves and avoid inhaling the powder), because they may be toxic and/or allergenic.

5 The general procedure outlined is appropriate for cloning all virus CP genes, but since each virus gene has a unique nucleotide sequence, the restriction enzyme sites used will differ In the example cited, the CP gene is excised with a small

amount of flanking sequence, necessitated by the absence of restriction enzyme sites at the N- and C-termini of the gene. If cloning of only the CP gene sequence is required, it will usually be necessary to engineer appropriate restriction sites, either by mutagenesis *(24)* or by PCR amplification of the required fragment with mutagenic primers, in which case it is advisable to sequence the PCR fragment Such mutagenesis should not be necessary if the aim is to engineer resistance, rather than identify the resistance mechanism; but always ensure that a translation initiation codon is not produced between the promoter and the CP initiation codon For genes that are normally expressed as a polyprotein (e g , potyvirus genes), an AUG initiation codon within a Kozak consensus may need to be added for CP expression *(25)*; for those CP genes that are not the 3'-terminal gene (e g., in comoviruses), a translational terminator should be inserted at the appropriate site

The amount of DNA to be digested will also vary, depending on how large a proportion of the construct the CP gene occupies For example, a 1-kb CP gene cloned into a pUC-based plasmid (of approx 3-kb size) represents about 25% of the total DNA concentration, thus a maximum of 2 5 μg of CP gene DNA may be released from 10 μg of the construct It is wise to assume that up to 50% of the total gene fragment may be lost during the extraction procedure and always to start with more construct DNA than is strictly necessary

6. A 1% agarose gel is suitable for most CP gene fragments, the buffer used will depend on the gel purification procedure used Many commercial kits are available for gel purification, in addition to the techniques described in Chapters 27, 33, and 43. **Note:** Ethidium bromide is carcinogenic, use gloves when handling it and limit exposure by purchasing preweighed tablets or solutions (Sigma, Poole, Dorset UK). To avoid breakage of the CP DNA, expose it to UV irradiation for the minimum time, and if possible use the preparatory option on the transilluminator, rather than the analytical setting. **Note:** Ultraviolet light may burn exposed skin and damage eyes, always wear appropriate protective gloves and glasses and/or face visors
7. The restriction enzyme sites used will depend on the sequence of the CP gene being used Gel purification of the cut vector will minimize the inclusion of any uncut vector and remove the small *Bam*HI-*Sac*I fragment If the vector is digested with a single enzyme, remove the 5' phosphate using calf intestinal phosphatase (*see* Chapter 27). It is useful to do this dephosphorylation step, even when two different enzymes have been used, as it will prevent religation if one of the enzymes has not completely digested the vector DNA Dephosphorylation is especially advisable when the vector does not have color selection to facilitate identification of insert-containing colonies
8 Estimate the DNA concentration of the vector and CP fragments by comparing their fluorescence following gel electrophoresis and ethidium bromide staining with that of a marker of known concentration For the ligation, include two tubes· one containing 100 ng vector, the other 100 ng vector plus ligase, to monitor the success of the vector digestion and dephosphorylation

9 As with other plasmids, the antibiotic will depend on the vector used Where *lacZ* color selection is possible (*see* Chapter 27), include X-gal and IPTG in the medium.

10. In the present example, the enzymes used should be *Bam*HI and *Sac*I Alternatively, the presence of the insert may be confirmed by PCR amplification using primers able to hybridize to sequences within the CP gene or within the 35S CaMV promoter and the nos terminator More details of the latter primers are shown in **Subheading 3.3.2.**, and PCR screening is described in Chapters 28, 41, 42, and 48.

11 *A. tumefaciens* may normally be grown in LB agar containing appropriate antibiotics (e g , rifampicin for strains LBA4404 and pGV3850, which carry the resistance gene on their chromosome) For cells required for electroporation, YEP medium should be used and they should be grown to an absorbance of 0 5–1 0 *Agrobacterium* should be grown between 25 and 30°C, temperatures higher than this may result in loss of the Ti plasmid. A shaking speed similar to that used for *E coli* (e g , 200–250 rpm) is acceptable

12 The DNA concentration in the mix should be approx 1 µg/40 µL cells It is not necessary to prepare highly purified DNA; clean miniprep DNA prepared by the alkaline lysis method ***(19)*** and resuspended in sterile distilled water is suitable If excess salt is present in the DNA solution, sparking may occur during the electroporation and this will markedly reduce the transformation efficiency If such problems are routinely encountered, remove the salt using proprietary clean-up columns or ethanol precipitation.

13 The gene pulser should be set to 25 µF capacitance and 2 5 kV charge, the pulse controller resistance to 200Ω Successful transformations have also been obtained with 400Ω

14 Include the antibiotics appropriate to the plasmids/strains used In this example, use kanamycin (50 µg/mL) for pRK2013 and pROKII, and rifampicin (50 µg/mL) for LBA4404 Cultures in exponential phase will give the greatest number of transconjugant colonies, but stationary phase cultures may be used.

15. Washing the cells removes the antibiotics that might kill the bacteria prior to the conjugation

16 Pipet the bacteria carefully onto the filters and do not move the plates until the filters have dried If the suspension runs off, the bacteria can be collected using the disk just before transfer to water If a laminar flow cabinet is not available, the mixed culture may be centrifuged for 5 min in a microcentrifuge, the supernatant removed, and the pellet placed over the filter or plated directly onto the agar Control filters, of each culture alone, should also be prepared

17 It is normally suggested that bacteria be resuspended and diluted in 10 m*M* $MgSO_4$, but sterile distilled water can be used provided that plating is done without undue delay

18 None of the commonly used *E coli* strains are resistant to rifampicin; thus, only *Agrobacterium* should grow on these plates Kanamycin selects for the presence of pROKII sequences in transconjugants, the pRK2O13 plasmid does not repli-

cate in *Agrobacterium* Plating of the original bacterial cultures will determine that none are able to grow on the plates containing both antibiotics. If required, transconjugants can be confirmed as *Agrobacterium* by their ability to metabolize 3-keto-lactose, as described by Bernaerts and Deley ***(26)*** To do this, bacteria are grown for 2–3 d on lactose yeast-medium agar plates (10 g lactose, 1 g Difco yeast extract and 20 g LabM agar per 1 L) and the plates flooded with Benedicts reagent. (Dissolve 173 g sodium citrate and 100 g anhydrous sodium carbonate in 850 mL water, then add, while stirring, to 17 4 g copper sulfate dissolved in 100 mL water Make to 1 L with water, it is not necessary to sterilize this reagent ) A yellow color reaction in the medium around the streak confirms the cultures to be *Agrobacterium* This method is suitable for all Biotype I strains of *Agrobacterium* (this includes all strains commonly used for plant transformation)

19 *Agrobacterium* cultures survive for only a short time (often less than 1 mo) on LB agar plates at 4°C, and therefore the transconjugant should be stored at –70°C To do this, prepare an overnight culture of the selected colony, as described in **Subheading 3.2.1.** Mix equal volumes of sterilized glycerol and bacteria to give a 50% glycerol solution Ensure that the contents of the tube are well mixed, then store at –70°C Using this glycerol concentration, the bacteria may be sampled without any need for thawing, because the culture remains slightly soft. Cultures stored in this way have remained viable for the past 8 yr in our laboratory. Storage at –20°C is suitable for times of less than 1 yr

20. Many binary vectors are based on low-copy-number replicons, and therefore the host bacteria should always be grown under appropriate antibiotic selection (in this case, kanamycin)

21. For the present example, *Bam*HI plus *Sac*I digestion will release a 657 bp (CP gene) fragment; *Hin*dIII plus *Eco*RI will release an approx 2-kb fragment, including the promoter, CP, and terminator The DNA obtained from *Agrobacterium* is often less clean than that obtained from *E coli*, so that two to five times the usual amount of enzyme should be added and digestion increased to 4–16 h (increase the reaction volume, if necessary) For controls, the original binary vector DNA, plus and minus the insert, should also be digested Calculate the fragment sizes by comparison with mol-wt markers

22. The CP gene fragment prepared in **Subheading 3.1., step 2** can be used for preparing the probe. Thoroughly check transconjugants prior to plant transformation, because rearrangement and recombination events may take place during the cloning procedure and multimeric copies of the CP fragment may be inserted into the vector

23 The primers described here are appropriate for the analysis of all constructs that use the CaMV 35S promoter and the nos terminator They are sited in regions conserved in all CaMV strains (S Covey, personal communication) and nos regions analyzed to date

24. Compare the size of the insert with a CP fragment (e.g., that prepared in **Subheading 3.1., step 2**). Alternative primers may be used, commonly, these have homology with the inserted viral gene sequence or T-DNA border sequences In

these cases, the reaction conditions should be calculated as described in Chapters 28, 41, 42, and 48. *Agrobacterium* colonies without an insert should be used as a control

## References


1 Heie, Y., Ohta, S , Komari, T , and Kumashiro, T (1994) Efficient transformation of rice (*Oryza sativa* L ) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA *Plant J* **6,** 271–282

2 de Framond, A , Barton, K A , and Chilton, M -D (1983) Mini Ti a new vector strategy for plant genetic engineering *Biotechnology* **1,** 262–269

3 Zambryski, P , Joos, H , Genetello, C , Leemans, J., Van Montagu, M., and Schell, J (1983) Ti-plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity *EMBO J* **2,** 2143–2150.

4. Herrera-Estrella, L and Simpson, J. (1988) Foreign gene expression in plants, in *Plant Molecular Biology A Practical Approach* (Shaw, C H , ed ), IRL, Oxford, UK, pp 131–160

5 Nagel, R , Elliott, A , Masel, A , Birch, R G , and Manners, J M (1990) Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes FEMS Microbiol Letts* **67,** 325–328

6 Holsters, M , De Waele, D , Depicker, A , Messens, E , Van Montagu, M , and Schell, J (1978) Transfection and transformation of *Agrobacterium tumefaciens Mol Gen Genet* **163,** 181–187

7 Schledzewski, K and Mendel, R R (1994) Quantitative transient gene expression comparison of the promoters for maize polyubiquitinl, rice actin1, maize-derived *Emu* and CaMV 35S in cells of barley, maize and tobacco *Transgenic Res* **3,** 249–255

8. Grant, J E , Cooper, P A., McAra, A. E., and Frew, T. J (1995) Transformation of peas *(Pisum sativum* L ) using immature cotyledons *Plant Cell Rep* **15,** 254–258

9. Bevan, M W (1984) Binary *Agrobacterium* vectors for plant transformation *Nucleic Acids Res* **12,** 8711–8721

10. Quemada, H D., Gonsalves, D., and Slightom, J L. (1991) Expression of coat protein gene from cucumber mosaic virus strain C in tobacco protection against infections by CMV strains transmitted mechanically or by aphids *Phytopathology* **81,** 794–802.

11 Namba, S , Ling, K., Gonsalves, C , Slightom, J L , and Gonsalves, D (1992) Protection of transgenic plants expressing the coat protein gene of watermelon mosaic virus II or zucchini yellow mosaic virus against six potyviruses *Phytopathology* **82,** 940–946

12. Ling, K , Namba, S , Gonsalves, C , Slightom, J L , and Gonsalves, D (1991) Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene *Biotechnology* **9,** 752–758.

13 Hooykaas, P. J J and Schilperoort, R. A. (1992) *Agrobacterium* and plant genetic engineering *Plant Mol Biol* **19,** 15–38

14 Yie, Y, Zhao, F., Zhao, S Z, Liu, Y Z, Liu, Y L, and Tien, P (1992) High resistance to cucumber mosaic virus conferred by satellite RNA and coat protein in transgenic commercial tobacco cultivar G-140 *Mol Plant–Microbe Interact* **5**, 460–465.

15 Ditta, G, Stanfield, S., Corbin, D., and Helinski, D R (1980) Broad host range DNA cloning system for Gram-negative bacteria· construction of a gene bank of *Rhizobium meliloti Proc Natl Acad Sci USA* **77**, 7347–7351

16. Frisch, D. A., Harris-Haller, L W, Yokubaitis, N T, Thomas, T L, Hardin, S H, and Hall, T. C (1995) Complete sequence of the binary vector Bin 19 *Plant Mol Biol* **27**, 405–409

17 van Engelen, F A, Molthoff, J W, Conner, A J, Nap, J-P, Pereira, A, and Stiekema, W J (1995) pBINPLUS· an improved plant transformation vector based on pBIN19 *Transgenic Res* **4**, 288–290

18. Jones, J D. G., Shlumukov, L, Carland, F, English, J, Scofield, S R, Bishop, G J., and Harrison, K (1992) Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants *Transgenic Res* **1**, 285–297

19 Sambrook, J, Fritsch, E F, and Maniatis, T (1989) *Molecular Cloning A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

20 Lazo, G R, Stein, P A, and Ludwig, R. A (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium BioTechnology* **9**, 963–967

21 Hood, E. E., Gelvin, S B, Melchers, L S, and Hoekema, A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants *Transgenic Res* **2**, 208–218

22. Grumet, R (1994) Development of virus-resistant plants via genetic engineering *Plant Breeding Rev* **12**, 47–79

23 Boulton, M I and Davies, J W (1990) Monopartite geminiviruses markers for gene transfer to cereals *Aspects Appl Biol* **24**, 79–86

24 Kunkel, T A, Roberts, J D, and Zakour, R A (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection *Methods Enzymol* **154**, 367–382

25 Dougherty, W G and Carrington, J C (1988) Expression and function of potyviral gene products *Annu. Rev Phytopathol* **26**, 123–143

26 Bernaerts, M J and De Ley, J (1963) A biochemical test for crown gall bacteria *Nature (London)* **197**, 406,407

# 36

# Potato Transformation

**Lee Rooke and Keith Lindsey**

## 1. Introduction

Potato is the world's fourth most important food crop, being surpassed in total production only by wheat, corn, and rice. Improved resistance to disease and pests during growth and storage is therefore of significant economic importance.

Cultivated varieties of *Solanum tuberosum* L. are tetraploid and exhibit a high level of genetic heterozygosity, which imparts vigor and high tuber yield to the cultivar. Improvement by conventional breeding is complicated because of segregation of the important characteristics and traits among the progeny. Genetic engineering offers the opportunity to introduce genes of interest and value into a cultivar without altering the commercially desirable phenotype Our own interest in potato transformation is in using T-DNA-mediated promoter trapping to identify tissue-specific genes in this species *(1)*.

Potato is one of the few crop species readily susceptible to infection by the soil-borne bacterium *Agrobacterium tumefaciens*, the causative agent of Crown Gall disease. This infection represents a natural gene vector system in which genetic information is transferred from the bacterium and integrated into the plant nuclear genome, where the transgenes can be expressed. The system has been exploited to genetically engineer plants by eliminating the oncogenic genes from the transferred DNA (T-DNA). Specific genes introduced between the T-DNA border repeats can then be transferred into plant cells without being accompanied by tumor formation (**ref. *2***; *see* Chapter 35). Transformed plant cells are directly selected for in tissue culture by the inclusion within the transferred DNA of a marker gene that confers resistance to an antibiotic or herbicidal compound *(3)*. Efficient transformation must be subsequently followed by reproducible regeneration of whole plants: Manipulation of hormone com-

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

binations in the culture medium can be used to induce multiplication of undifferentiated callus prior to induction of shoot formation (*see* **Note 1**).

*Agrobacterium*-mediated transformation has been employed successfully with a number of different potato cultivars ***(4–6)***, using leaf ***(4,7)***, stem ***(7–9)***, or tuber disks ***(5,6)*** as explant tissue Continuous availability of healthy, vigorously growing leaf and stem material by micropropagation of shoot cultures in vitro is a major advantage over the use of field-grown tubers, for which age is a critical factor in their transformability.

This chapter details a transformation protocol for the cultivar Désirée, using in vitro cultured stem explants, that is rapid and efficient Désirée is amenable to tissue culture and has a higher capacity for transformation and regeneration than many other potato cultivars, which may require alternative or additional conditions (*see* **Notes 1** and **2**)

## 2. Materials

### 2.1. Transformation Vector System

1 An avirulent strain of *A tumefaciens*, for example, LBA4404, containing the disarmed helper plasmid pRAL4404 ***(10)***
2 A binary vector, such as pBin19 ***(11)***, with the gene(s) to be transferred integrated into the multiple cloning site between the T-DNA border repeats

### 2.2. Bacterial Culture Media

1 LB (Luria-Bertani) broth 10 g/L bacto-tryptone (Difco, Detroit, MI), 10 g/L sodium chloride, 5 g/L bacto-yeast extract (Difco), make up in deionized water and alter to pH 7.0 with 1*N* NaOH.
2 LB agar composition as for LB broth; solidify with 15 g/L bacto-agar (Difco)
Sterilize the media by autoclaving (121°C, 15 min) and add the appropriate antibiotics when it has cooled to 45–55°C The *A tumefaciens* strain LBA4404 is resistant to rifampicin (use at 100 mg/L medium), and the binary vector pBin19 confers bacterial and plant cell resistance to kanamycin (for bacterial medium, use at 50 mg/L)
3 Incubator and shaker set at 29°C for *Agrobacterium* cultures

### 2.3. Plant Material

1 Virus-free in vitro stock of the potato cultivar To maintain healthy material, subculture every 4–6 wk by transferring the top 1.5 cm of the shoot (containing the apical meristem) onto fresh MS30 medium in either 30 mL sterile plastic tubes, glass culture tubes (Sigma, St Louis, MO), or Magenta vessels (Sigma)
2 Growth incubator or room, set at 25°C with a 16-h photoperiod, and a photon flux density of approx 50 μmol/$m^2$/s, from fluorescent lighting

### 2.4. Plant Culture Media

1. MS liquid Murashige and Skoog basal medium (**ref.** ***12***; Sigma) with 30 g/L sucrose, make up in deionized water and adjust to pH 5 9 with 1*N* KOH

2. MS30 medium. Composition as for MS liquid; solidify with 8 g/L bacto-agar (Difco)
3 Callus-inducing medium MS30 supplemented with 0.186 mg/L NAA, 2.25 mg/L BAP, 10 mg/L $GA_3$, 50 mg/L kanamycin, and 200 mg/L cefotaxime
4. Shoot-inducing medium: Composition as for callus-inducing medium, but omitting the NAA.

Sterilize all media by autoclaving (121°C, 15 min). Because many antibiotics and hormones are heat-labile, add to the medium after autoclaving, when it has cooled to approx 45–55°C

## 2.5. Antibiotic Stocks

1 Cefotaxime bought commercially as Claforan (Roussel), dissolve in deionized water to a concentration of 100 mg/mL, filter-sterilize (0 2-μm filter), and store at –20°C Cefotaxime is not stable in the light and breaks down rapidly, therefore medium containing cefotaxime should be prepared immediately before use, and not stored.
2 Kanamycin sulfate (Sigma)· Dissolve in deionized water to a concentration of 50 mg/mL, filter-sterilize, and store at –20°C
3 Rifampicin (Sigma) Dissolve in methanol to a concentration of 50 mg/mL and store at –20°C

**Caution:** Since these chemicals can be harmful, wear suitable protective clothing when preparing stocks

## 2.6. Hormone Stocks

1 BAP, 6-benzylaminopurine (Sigma) Dissolve in 1*N* NaOH to a concentration of 4 5 mg/mL, filter-sterilize (0 2-μm filter), and store at 0–5°C
2 $GA_3$, gibberellic acid (Sigma)· Dissolve in deionized water to a concentration of 20 mg/mL, filter-sterilize, and store at 0–5°C
3. NAA, 1-naphthalene acetic acid (Sigma) Dissolve in 1*N* NaOH to a concentration of 1 86 mg/mL, filter-sterilize, and store at 0–5°C.

## 2.7. Miscellaneous

1. Laboratory sealing film and Micropore tape (3M, Loughborough, UK)
2 Presterilized 9-cm plastic Petri dishes (Sterilin, Stone, UK)
3 Inoculating loop and tissue culture implements.

# 3. Methods

This method is based on that described by Twell and Ooms *(8)*. All tissue culture manipulations must carried out under sterile conditions in a laminar flow hood.

1. Streak the *Agrobacterium* onto LB agar plates containing rifampicin and kanamycin, so that a confluent bacterial lawn will be produced, and incubate at 29°C for 48 h
2. Prepare the potato material by cutting stems from the in vitro-grown plants into internodal sections approx 0 5–1 0 cm in length Ensure the sections are devoid of leaves and axillary buds.

3 Stand the stem sections vertically in MS30 medium, poured in Petri dishes, and smear the apical end with *Agrobacterium* taken from the LB plate, using a sterile inoculating loop (*see* **Note 3**)
4 Seal the Petri dishes with sealing film (*see* **Note 4**) and allow to cocultivate for 48 h (for incubation conditions, *see* **Subheading 2.3.**)
5 Following cocultivation, transfer the explants to callus-inducing medium, the sections can now be laid horizontally (*see* **Note 5**)
6 Seal the Petri dishes with Micropore tape (*see* **Note 6**) and incubate as before
7 Green callus will develop at the inoculated, apical end of the stem section within 2–4 wk Transfer the callus, or the whole explant, to shoot-inducing medium and seal the Petri dishes with Micropore tape
8 When regenerated shoots have reached a height of 0 5–1 0 cm, transfer them onto MS30 medium to induce rooting The medium should be supplemented with both cefotaxime and kanamycin (*see* **Note 7**)
9 Rooted shoots are maintained on MS30 medium supplemented with kanamycin in the same way as the potato stocks (*see* **Subheading 2.3.**) and tested by PCR and Southern analysis to confirm transformation (*see* Chapters 41–43)

Regeneration of shoots from the callus should occur within 2–6 wk, and the entire process of transformation and regeneration of rooted transgenic plantlets can be accomplished in 2–3 mo.

It is advisable to include a full range of controls with each transformation experiment carried out (*see* **Note 8**).

## 4. Notes

1 A prerequisite of plant transformation is a reliable, efficient regeneration protocol, because the transformation procedure reduces the regeneration efficiency significantly. Different cultivars have their own individual preferences of hormone combinations and concentrations for optimal regeneration ***(6,13)*** An appropriate ratio of cytokinin (BAP) to auxin (NAA) in the medium induces plant cells to undergo proliferation into undifferentiated callus, and, by decreasing or removing auxin, the cells are induced to differentiate into shoots Inclusion of gibberellic acid ($GA_3$) can promote stem elongation The callus phase needs to be kept to a minimum to reduce the risk of somaclonal variation (phenotypic variation, either genetic or epigenetic in origin)
2 Different cultivars vary in their amenability to transformation and regeneration As well as variation in hormone levels and combinations (*see* **Note 1**), the transformation protocol can include preconditioning the explants before cocultivation with the bacterial cells ***(7,13)***, addition of silver nitrate in the growth medium to obtain efficient shoot regeneration ***(4)***, and the use of a nurse or feeder layer ***(5,9)*** During cocultivation, the explants are incubated on filter paper above a feeder layer of plant cell suspension, which is in contact with the culture medium. This requires the availability of an exponentially growing cell suspension (normally tobacco, petunia, or potato) Acetosyringone is the prominent inducer of

the bacterial virulence genes, involved in T-DNA transfer, released in solanceous plant tissue following wounding ***(2)***, and can be included in bacterial growth medium to increase the frequency of T-DNA transfer ***(6)***. With certain cultivars the choice of explant, vector, and *Agrobacterium* strain may prove important in developing an efficient transformation protocol

3 Both ends of the stem section can be smeared with bacteria and the explant laid horizontally on the medium Incubate 15–20 explants per Petri dish

An effective alternative to this method is to immerse the stem sections, and leaves (cut into halves) in a liquid culture of *Agrobacterium*. Grow an overnight culture of *Agrobacterium* in LB broth (remember to include antibiotics) at 29°C, with shaking at 200 rpm, and pellet by centrifugation at 3000*g* for 5 min Resuspend the cells in twice the volume of MS liquid (this dilution varies, from 2- to 100-fold, with different protocols, *see* **refs. *5***, ***6***, and ***13***) and immerse the explants for 10 min. Briefly blot the explants on sterile filter paper, being careful not to let them dry out, and transfer them to MS30, making sure they are in close contact with the culture medium The remainder of the protocol is exactly the same as that described, and regeneration, from callus at the cut explant surfaces, occurs within the same time-scale Because in vitro-grown leaves are small, leaves taken from soil-grown plants can be used, but need to be surface-sterilized A sterile cork-borer is a quick way of cutting uniformly sized leaf disks

4 Sealing the plates maintains humidity, which is thought to enhance infection

5 Decrease the number of explants to 5–7 per Petri dish. Callus-inducing medium is supplemented with kanamycin to select for transformed plant cells, and with cefotaxime to kill the *Agrobacterium* The level of kanamycin (50 mg/L) is above the concentration on which nontransformed tissue is able to grow

6 Micropore tape allows gas exchange and prevents the plates from becoming too humid If the plant tissues become overrun with *Agrobacterium*, then transfer them to fresh plates (cefotaxime is degraded rapidly in light) and leave the plates unsealed If this becomes a serious problem, the explants can be washed in a cefotaxime solution

7 Either take one shoot per callus or identify shoots obtained from the same callus, because they may be clonal in origin. It is important to include cefotaxime and kanamycin in the medium during rooting, to help prevent bacterial regrowth that can interfere with subsequent analyses, and to ensure selection against escapes Occasionally, shoots that are not transformed or do not express the foreign DNA can survive on medium containing selection. Roots are relatively sensitive to antibiotics, and inclusion of selection in the rooting medium should prevent root formation on nontransformed shoots.

8 Controls should be included with each experiment. A full set of controls would consist of inoculated explants incubated on culture medium lacking antibiotic selection, explants inoculated with the *Agrobacterium* strain (no binary vector) incubated on medium with and without antibiotic selection, and uninoculated explants incubated on medium with and without antibiotic selection and cefotaxime

## References


1 Lindsey, K , Wei, W , Clarke, M C , McArdle, H F , Rooke, L M , and Topping, J F (1993) Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants *Transgenic Res.* **2,** 33–47

2 Hooykaas, P J J and Schilperoort, R. A (1992) *Agrobacterium* and plant genetic engineering *Plant Mol Biol* **19,** 15–38

3 Bevan, M W , Flavell, R. B , and Chilton, M -D (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation *Nature* **304,** 184–187

4 De Block, M (1988) Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens Theor Appl Genet* **76,** 767–774

5 Sheerman, S and Bevan, M W (1988) A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors *Plant Cell Rep* **7,** 13–16

6 Hoekema, A , Huisman, M J., Molendijk, L , van den Elzen, P J. M , and Cornelissen, B J C (1989) The genetic engineering of two commercial potato cultivars for resistance to potato virus X. *Biotechnology* **7,** 273–278

7 Visser, R G F., Jacobsen, E , Hesseling-Meinders, A , Schans, M J , Witholt, B., and Feenstra, W J (1989) Transformation of homozygous diploid potato with an *Agrobacterium tumefaciens* binary vector system by adventitious shoot regeneration on leaf and stem segments *Plant Mol Biol.* **12,** 329–337

8 Twell, D and Ooms, G (1987) The 5' flanking DNA of a patatin gene directs tuber specific expression of a chimaeric gene in potato *Plant Mol Biol* **9,** 365–375

9 Newell, C A , Rozman, R , Hinchee, M A., Lawson, E C , Haley, L , Sanders, P , Kaniewski, W , Tumer, N. E , Horsch, R. B , and Fraley, R T (1991) *Agrobacterium*-mediated transformation of *Solanum tuberosum* L cv "Russet Burbank" *Plant Cell Rep* **10,** 30–34

10 Ooms, G , Hooykaas, P J J , Van Veen, R J. M., Van Beelen, P , Regensburg-Tuink, T. J G , and Schilperoort, R A (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region *Plasmid* **7,** 15–29

11 Bevan, M (1984) Binary *Agrobacterium* vectors for plant transformation *Nucleic Acids Res* **12,** 8711–8721

12 Murashige, T and Skoog, F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures *Physiol Plant* **15,** 473–497

13 Visser, R. G. F. (1991) Regeneration and transformation of potato by *Agrobacterium tumefaciens*, in *Plant Tissue Culture Manual* (Lindsey, K , ed ), Kluwer, Dordrecht, pp. B5/1–9.

# 37

# Transformation of Tomato

**Artur J. P. Pfitzner**

## 1. Introduction

Since 1986, when Beachy and coworkers *(1)* first published a protection of transgenic tobacco plants expressing the tobacco mosaic virus (TMV) coat protein (CP) against TMV infection, transgenic plants have become an important tool in plant virology. Coat protein transgenic plants have been used successfully to obtain protection against many different plant viruses *(2)*. In addition, transgenic plants expressing viral gene products can be used to study the function of one particular gene in the viral life cycle or in the interaction with specific host plants.

Most of these experiments have been conducted with tobacco as a model plant. A plant species of great commercial importance, which is affected by many viral diseases, is tomato (*Lycopersicon esculentum* Mill.). Since tomato can be transformed using *Agrobacterium*, it is a good choice as an alternative to tobacco for studying viral gene function in transgenic plants However, in contrast to tobacco, there is a considerable variation in the tissue culture performance of different tomato cultivars. The method described here was optimized in our laboratory for the transformation of greenhouse tomato cultivars like Craigella and Moneymaker. It is based on protocols developed by Filatti and coworkers *(3)* and by Smith et al. *(4)*.

## 2. Materials

1. *Agrobacterium* strains For transformation of tomato the *Agrobacterium tumefaciens* strain LBA4404 was used, which harbors the severely deleted Ti plasmid pAL4404. This Ti plasmid serves as a helper for the transfer of the T-DNA from a second (binary) plasmid that contains the selectable marker and the gene of interest We use the binary plasmid, pBin19 *(5)*, and derivatives of this plasmid.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

2 Tomato cultivars: The transformation procedure described here was optimized for *L. esculentum* Mill. cv. Craigella and tested for a variety of mutants in the Craigella background. Seeds of this tomato variety can be obtained from Horticulture International, Wellesbourne, UK. Another tomato cultivar, which was frequently used for the generation of transgenic plants, is *L. esculentum* Mill. cv. Moneymaker. This tomato variety is very popular and can be obtained from many local seed companies.

3 5X Min A salts: Dissolve 52.5 g $K_2HPO_4$, 22.5 g $KH_2PO_4$, 5.0 g $(NH_4)_2SO_4$, and 2.5 g sodium citrate x $2H_2O$ in 1 L $ddH_2O$, and autoclave.

4 Min A medium: Mix 10 mL 5 × Min A salts and 40 mL $ddH_2O$. Autoclave and then add 50 µL 20% $MgSO_4$ solution (filter-sterilized) and 500 µL 20% glucose solution (filter-sterilized).

5 Rifampicin stock solution: Dissolve 50 mg of Rifampicin in 1 mL DMSO.

6 Kanamycin stock solution: Dissolve 25 mg of kanamycin in 1 mL $ddH_2O$ and filter-sterilize the solution (*see* **Note 1**).

7 Hygromycin stock solution: Dissolve 30 mg of hygromycin B in 1 mL $ddH_2O$ and filter-sterilize the solution.

8 Acetosyringone stock solution: 10 m*M* acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) in ethanol.

9 Zeatin riboside stock solution: Dissolve 10 mg of zeatin riboside in 10 mL $ddH_2O$ and filter-sterilize the solution.

10 Carbenicillin stock solution: Dissolve 1 g of carbenicillin in 4 mL of $ddH_2O$ and filter-sterilize the solution.

11 100 × B5 vitamins: Dissolve 10 mg of nicotinic acid, 100 mg of thiamine hyddrochloride, 10 mg of pyridoxine hydrochloride, and 1 g of myo-insitol in 100 mL of $ddH_2O$, filter-sterilize, and freeze at –20°C in aliquots.

12 MSO medium: Dissolve 4.6 g of MS salts and 30 g of sucrose in 1 L $ddH_2O$, adjust the pH to 5.4 with KOH, and autoclave, then add 10 mL of the 100X B5 vitamin stock solution.

13 MSOZR medium: Prepare MSO medium and add 0.5 mL acetosyringone stock solution and 2 mL zeatin riboside stock solution. For the preparation of solid medium, add 6 g of agar per 1 L medium before autoclaving.

14 Agrobacterium induction medium: Mix the following:
   a 5 mL 2X MS salt solution (9.2 g in 1 L $ddH_2O$, pH 5.4).
   b 1.25 mL Sodium phosphate buffer (100 m*M*, pH 5.4)
   c 5 µL Acetosyringone stock solution (10 m*M*)
   d 20 µL Rifampicin stock solution (50 mg/mL)
   e 20 µL Kanamycin stock solution (25 mg/mL)
   Then add $ddH_2O$ to a final volume of 10 mL.

15 Selective shoot regeneration medium: Dissolve 4.6 g of MS salts and 30 g of sucrose in 1 L of $ddH_2O$, add 6 g of agar, adjust the pH to 5.7 with KOH, and autoclave. Cool the medium down to 50°C in a water bath and add 10 mL of 100X B5 vitamin stock solution, 2 mL of zeatin riboside stock solution (1 mg/mL), 4 mL of kanamycin stock solution (25 mg/mL), and 2 mL of carbenicillin stock

solution (250 mg/mL). Pour the medium into sterile Petri dishes or into sterile plant containers and let it solidify at room temperature

16 Selective root regeneration medium: Dissolve 4 6 g of MS salts and 10 g of sucrose in 1 L of $ddH_2O$, add 4 g of agar, adjust the pH to 5 7 with KOH, and autoclave Cool the medium down to 50°C in a water bath Then add 10 mL of 100X B5 vitamin stock solution, 2 mL of kanamycin stock solution (25 mg/mL), and 2 mL of carbenicillin stock solution (250 mg/mL) Pour the medium into sterile plant containers and let it solidify at room temperature

17 Germination medium Dissolve 4 6 g of MS salts (Sigma) in 1 L of agar and adjust the pH to 5 7 with KOH Autoclave and cool down to 50°C. Pour the medium into sterile plant containers and let it solidify at room temperature

## 3. Methods

1 Sterilize tomato seeds for 30 s in ethanol, for 20 min in 1 5% NaOCl, 0.1% Tween-20, and then wash three times in sterile water The seeds are then seeded on sterile germination medium and are incubated at room temperature in the dark After 3–5 d, most seeds have germinated. About 20–30 seeds are incubated in one sterile plant container (7 × 10 cm) For one transformation experiment, 50–60 seedlings are needed

2 After germination, the sterile plant containers are transferred to a plant growth chamber with a temperature of 25°C and with a photoperiod of 16 h light and 8 h dark. After 8 d, the cotelydons have grown to the maximal size and can be used for transformation

3 Thirty-six hours before the harvest of the cotyledons, the *Agrobacterium* strain containing the plasmid to be tranferred into the tomato genome, e g , pBin19, is inoculated in 5 mL Min A medium For pBin19, add 50 μg/mL kanamycin and 100 μg/mL rifampicin. The *Agrobacterium* culture is incubated in a bacteria shaker at 30°C

4. The cotyledons of the sterile tomato seedlings are cut off and the tips of the leaves are removed Then, the cotyledons are cut in half and placed upside up on plates with MSOZR medium containing 0 6% agar The Petri dishes are sealed with parafilm and incubated in a plant growth chamber (25°C, photoperiod of 16 h light/8 h dark)

5. Forty-eight hours after starting the *Agrobacterium* culture, the bacteria are harvested by centrifugation at 4300*g* and resuspended in 3 mL of sterile *Agrobacterium* induction medium (*see* **Note 2**) The *Agrobacteria* are now incubated for another 12 h at 30°C in a bacteria shaker

6. After 12 h of incubation in the induction medium, the *Agrobacteria* are pelleted at 4300*g*, resuspended in 3 mL of MSO medium, and diluted by adding 15 mL MSO medium (*see* **Note 3**) The cut pieces of the tomato cotyledons are removed from the MSOZR plates, dipped into the *Agrobacteria* solution, blotted dry on sterile filter paper, and placed back on the same MSOZR plates

7 The MSOZR plates are sealed again and incubated for 2 d in a plant growth chamber under the same conditions

8 After 2 d of incubation, the tomato cotyledon explants are transferred to plates with selective shoot regeneration medium and incubated in a plant growth chamber under the same conditions (*see* **Note 4**)
9 Every week, the cotyledon pieces are placed on fresh selective shoot regeneration medium (*see* **Note 5**). After about 4 wk, depending on the tomato cultivar, the first calli can be observed
10 Leaf pieces with calli, which have reached a size of ca 1–2 mm, are transferred individually to plant containers (7 × 10 cm) with selective shoot regeneration medium This procedure results in a much better growth of the calli (*see* **Note 6**)
11. After about 2 wk, shoots start to grow from the calli Shoots of ca 1 cm in size are cut off and placed on selective root regeneration medium in sterile plant containers (7 × 10 cm)
12. Within 1–2 wk, roots start to develop. Rooted shoots are now transferred to soil and further cultivated in the greenhouse (*see* **Note 7**)

## 4. Notes

1 Stock solutions are all filter-sterilized using a 0.45-µm Millipore filter and frozen in aliquots at –20°C
2 Acetosyringone and an acidic pH are important for the induction of the *vir* genes of *Agrobacterium.*
3 Since overgrowth of the tomato leaf pieces by *Agrobacteria* is a constant problem with tomato transformation, it is very important to dilute the *Agrobacterium* culture well enough
4. The transformation efficiency can be enhanced by a factor of about two by using feeder layers of suspension culture cells from tobacco In our hands, the feeder layers turned out to be a constant source of contaminations Therefore, we recommend omitting the feeder cells and following the above protocol carefully.
5 A weekly change of the medium is very important to keep the *Agrobacteria* down
6 It is also important to watch the calli very carefully If the calli start to produce phenolic compounds, the medium has to be changed immediately Otherwise, the calli may die within hours
7 Tomato plants can be easily multiplied by cuttings

## References

1 Powell Abel, P A , Nelson, R S , De, B , Hoffmann, N , Rogers, S G., Fraley, R T , and Beachy, R N (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene *Science* **232,** 738–743.
2 Beachy, R. N , Loesch-Fries, S , and Turner, N. E (1990) Coat protein mediated resistance against virus infection *Ann Rev Phytopathol* **28,** 451–474
3 Filatti, J. J., Kiser, J , Rose, B., and Comai, L (1987) Efficient transformation of tomato and the introduction and expression of a gene for herbicide tolerance, in

*Tomato Biotechnology* (Nevins, D J and Jones, R. A , eds ), Liss, New York, pp. 199–210.

4 Smith, C J S., Watson, C F , Ray, J , Bird, C. R , Morris, P. C , Schuch, W , and Grierson, D. (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* **334,** 724–726

5. Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation *Nucleic Acids Res.* **12,** 8711–8721

# 38

# Tobacco Transformation

**Jennifer F. Topping**

## 1. Introduction

The generation of genetically transformed plants is central to, and has indeed revolutionized, plant molecular biology. This is true for studies at both the fundamental and more applied levels of research. For researchers interested in unraveling the roles of specific genes in particular pathways of growth and development, the introduction into plants of foreign genes and gene promoters linked to reporter genes allows the detailed study of the temporal, spatial, and quantitative expression of plant genes and the activities of associated regulatory sequences.

It is not yet possible to transform many of the important crop species, and therefore so-called model plants species are used widely in transgenic research. A model plant species, for use in such studies, can be defined as one that can be efficiently and simply transformed with foreign DNA. Furthermore, the transformed cells or tissues must then be able to regenerate to produce fertile mature plants that produce transgenic seed.

Over the years, one particular dicot species that has emerged as an excellent model plant for transgenic studies is *Nicotiana tabacum* (Tobacco). One tobacco cultivar commonly used is *N. tabacum* cv. Petit Havana SR1 (commonly abbreviated to SR1); the methods described here are specifically for this variety. However, the methods are also applicable to other cultivars, such as Samsun and Xanthi. The most efficient and technically most simple method of transforming tobacco is to infect leaf explants with disarmed strains of the naturally occurring soil-borne bacterium *Agrobacterium tumefaciens*, which contains a disabled (nononcogenic) Ti plasmid ***(1)***. The gene construct to be transferred is integrated between the T-DNA borders of a binary vector ***(2)***, which is introduced into the *Agrobacterium*. Following inoculation, and under

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

suitable culture conditions, the leaf explants will readily regenerate transgenic plantlets which can then be potted out and grown to maturity

This chapter will describe and discuss the techniques involved in the establishment and maintainance of tobacco shoot cultures, which are source of leaf material for *Agrobacterium*-mediated transformation, the introduction of plasmids into *Agrobacterium* by triparental mating, the inoculation of leaf explants with *Agrobacterium*, and the subsequent selection and regeneration of the transformed material

Our laboratory has found it very convenient to use aseptic leaves from shoot cultures as the source material for transformations. However, it is possible to use greenhouse grown plants as a source of leaf material, provided that the leaves are free from pests and diseases, and that they are surface-sterilized prior to transformation.

## 2. Materials

### 2.1. Plant Growth Regulators and Antibiotics

1 Benzylaminopurine (BAP, Sigma, Poole, UK)· Make a stock solution of 1 mg/mL by dissolving the BAP in a minimum volume of dilute HCl (0.01*M*), and make up to the final volume with $ddH_2O$ The BAP solution can either be filter-sterilized through a 0.2-μm acrodisk and added to the autoclaved medium or it can be co-autoclaved with the medium The solution is stable for several months at –20°C

2. Kanamycin sulfate (Sigma). Make a stock solution of 100 mg/mL in $ddH_2O$ and filter-sterilize through a 0.2-μm acrodisk The solution is stable for several months at –20°C.

3 Cefotaxime. Cefotaxime can be purchased from Sigma, but it is cheaper if purchased from Roussel Laboratories (Uxbridge, UK) under the trade name Claforan Make a stock solution of 100 mg/mL in $ddH_2O$, filter-sterilize, and store in the dark at –20°C

4 Rifampicin (Sigma). Make up a stock of 20 mg/mL in methanol and store at –20°C

### 2.2. Plant Culture Medium

1. 1/2 MS10 (Germination medium) 2 2 g/L Murashige and Skoog basal medium (Sigma), 10 g/L sucrose, 8 g/L agar (Becton and Dickinson, Plymouth, UK) Adjust pH to 5.8 with 1*M* KOH, and autoclave (121°C, 20 min) The media can be stored for several months at room temperature
2. MS30· 4.4 g/L Murashige and Skoog basal medium (Sigma), 30 g/L sucrose, 8 g/L agar (Becton and Dickinson) Adjust pH to 5 8 with 1*M* KOH, and autoclave (121°C, 20 min) The media can be stored for several months at room temperature
3. Shooting medium 1: MS30, 1 mg/L BAP. Add the appropriate volume of the stock BAP solution to the molten MS30 when it has cooled to 50–60°C.

4. Shooting medium 2. MS30, 1 mg/L BAP, 100 mg/L kanamycin sulfate, 200 mg/L cefotaxime Add the appropriate volumes of the stock BAP and antibiotic solutions to the molten MS30 when it has cooled to 50–60°C
5 Rooting medium MS30 plus 200 mg/L cefotaxime and 100 mg/L kanamycin sulfate. Add the appropriate volumes of the antibiotic solutions to the molten MS30 when it has cooled to 50–60°C

## 2.3. Bacterial Culture Medium

1 Luria-Bertani (LB) broth· 10 g/L tryptone (Difco, Detroit, MI), 5 g/L yeast extract (Difco), 5 g/L NaCl Dissolve the solids, adjust the pH to 7 2 with 1*N* NaOH, and autoclave (121°C, 20 min) The broth can be stored at room temperature for several weeks.
2. LB agar. Make up LB broth as described above, then aliquot into bottles, add 15 g/L agar (Becton and Dickinson), and autoclave (121°C, 20 min) The agar can be stored at room temperature for several weeks

## 2.4. Bacterial Strains

1 *A tumefaciens* strain LBA4404 *(3)*
2. *Escherichia coli* helper strain pRK2013 *(4)*.

## 2.5. Miscellaneous Solutions

1 70% (v/v) Ethanol
2 Bleach solution. 10% (v/v) sodium hypochorite, 0 05% (v/v) Tween-20
3 Sterile $dH_2O$
4 10 m*M* $MgSO_4$

## 2.6. Plant Growth Containers

The seeds should be germinated and grown for 2–3 wk in deep 9-cm Falcon® Petri dishes (Becton and Dickinson, Loughborough, UK). The transformed shoots should be grown initially in 60-mL polypots (Northern Media, Loughborough, UK). The seedlings can then be transferred to larger (250–500 mL vol) vessels. Kilner jars are ideal, and plastic Magenta boxes are available from Sigma. In either case, there should be good ventilation to prevent the buildup of ethylene, which may inhibit plant growth *(5)* Therefore, the lids should not be tightened, or they can be removed completely and replaced with one half of a Petri dish and sealed with gas-permeable Micropore® tape (3M, St. Paul, MN).

## 2.7. Miscellaneous Materials and Equipment

1 Sterile filter paper
2 Sterile Petri dishes.
3. Sterile polypot or beaker
4 Scalpel

5 Forceps
6 Laboratory sealing film (Whatman)

## 3. Methods

### 3.1. Establishment and Maintainance of Tobacco Shoot Cultures

#### 3.1.1. Sterilization of Tobacco Seed

1. Working in a sterile flow cabinet, place the seeds in a flat bottomed tube or beaker
2 Immerse the seeds in 70% (v/v) ethanol for 20 s and agitate by gently swirling (*see* **Note 1**)
3 Tobacco seeds tend to float, so remove the ethanol using a sterile pipet by keeping the tip of the pipet close to the bottom of the beaker to suck off the ethanol, leaving the seeds at the bottom of the tube
4 Immerse the seeds in the bleach solution for 20 min (*see* **Note 2**) Agitate occasionally by swirling
5 Remove the bleach solution using a sterile pipet, as described in **step 3**, and wash the seeds five times in sterile distilled $H_2O$ (*see* **Note 3**) Leave a small volume of the final wash in the tube (0 1–0.3 mL)

#### 3.1.2. Germination of the Seeds

1 Using a sterile pipet, transfer the sterilized seeds in the final wash solution to a deep 9-cm Falcon Petri dish containing germination medium Tilt the plate and remove the excess $H_2O$ with the pipet. Gently spread the seeds out with the pipet and seal the dish with Micropore tape
2 Wrap the plates in aluminium foil, to exclude light, and leave at 4°C for 2–3 d to vernalize
3 Remove the foil and transfer the plates to a growth cabinet set at 22–25°C and with a light intensity of 50–200 μmol/$m^2$/s, supplied by Warmwhite® or Coolwhite® (Osram, Merseyside, UK) fluorescent tubes The light can be constant or long daylength. Germination will occur within 2–4 d

#### 3.1.3. Initiation and Subculture of Shoot Cultures

1 When the seedlings are 2–3 wk old and large enough to handle, transfer them using sterile forceps, in a sterile flow cabinet, to a larger vessel, for example, a 60-mL sterile polypot containing MS30 In about 2–3 wk, the plantlets will have three to four pairs of leaves and can be subcultured
2 To subculture the shoots, excise the apical bud and the newest pair of leaves from the plantet with a scalpel and embed the explant in MS30 in a Kilner jar or a similar vessel with a loose-fitting lid A second and perhaps third cutting can be made from the same plantlet, if necessary, remove a stem section containing a pair of leaves and, hence, axillary shoot meristems, and place in another Kilner jar (*see* **Note 5**) Continue to grow the shoot cultures under the same conditions as described above After 4–6 wk, the shoots will have produced roots and will have several pairs of fully expanded leaves. It is these fully expanded leaves that are used for transformation by *Agrobacterium*.

3 Shoots should be subcultured routinely every 4–5 wk into fresh MS30 (if the cultures become contaminated, *see* **Note 6**).

## 3.2. The Introduction of Plasmids into Agrobacterium by Triparental Mating

### 3.2.1. Setting Up Cultures of Parental Plasmids

1. Two days before the triparental mating, set up 10 mL LB broth + 100 mg/L rifampicin culture of LBA4404 Leave the culture to grow for 48 h at 28–30°C, with constant shaking
2 The day before the mating, set up a overnight culture of pRK2013 (in *E. coli* strain HB101) and an overnight culture of the *E coli* strain containing the construct to be transformed into the tobacco, each in 10 mL LB broth + 100 mg/L kanamycin sulfate. In our laboratory, we routinely use the binary vector Bin19 *(2)* transformed into XL1 blue cells *(6)*, supplied by Stratagene (Cambridge, UK) The Bin19 plasmid confers to bacterial and plant tissues resistance to kanamycin sulfate; therefore, add 100 mg/L of kanamycin sulfate to the overnight culture Grow both cultures at 37°C, with constant shaking

### 3.2.2. Mating Procedure

1 Take 100-µL aliquots from each 10-mL culture and mix together in a sterile 1 5-mL Eppendorf tube (store the remaining liquid cultures at 4°C)
2 Spin the cells down in a microcentrifuge (12,000*g* for 5 min)
3 Resuspend the pellet in 10 µL of 10 m*M* $MgSO_4$
4 Put the 10-µL droplet onto an LB agar plate and incubate overnight at 28–30°C During this time, mobilization of the plasmid occurs
5 Using a microbiological loop, streak out a patch of the bacteria onto an LB agar plate containing 100 mg/L kanamycin and 100 mg/L rifampicin At the same time, as a control, streak out each of the parental strains from **step 3** on a duplicate plate and incubate the plates overnight at 28–30°C. Only the LBA4404 harboring the Bin 19 vector should grow
6. Restreak for single colonies, if necessary.
7 Check by DNA restriction analysis that no rearrangements have occurred in the gene construct to be transformed into tobacco (*see* **Note 7**)

## 3.3. Inoculation of Leaf Explants with Agrobacterium

1 Working in a sterile environment, cut fully expanded leaves from 4- to 6-wk-old shoot cultures and place them in a sterile Petri dish
2 With a scalpel, remove the leaf midrib and the leaf edge and then cut the lamina into small pieces approx 1 $cm^2$ (*see* **Note 8**) Place 5–6 explants adaxial-side up on a plate of shooting medium 1 Prepare 20–30 explants for each construct
3 Seal the plates with laboratory sealing film and incubate the explants for 2 d under constant light at 22°C

4 On the day before the inoculation, set up a 75-mL LB broth culture of the LBA4404 strain harboring the DNA construct from a fresh overnight culture Add 100 mg/L of both kanamycin sulfate and rifampicin to the LB broth and incubate overnight at 28–30°C, with shaking (200 rpm)
5 Measure the optical density of the culture at 600 nm and dilute it, if necessary, with fresh LB broth to an $OD_{600}$ of 1 0 (*see* **Note 9**) Pour the culture into a sterile beaker or polypot
6 With sterile forceps, remove the leaf explants from the plates and immerse them in the bacterial suspension Mix by swirling and leave for 1 min
7 Remove the explants and blot them dry on sterile filter paper (*see* **Note 10**)
8 Replace the explants on the same shooting medium 1 plates, again adaxial-side up, seal the plates with laboratory sealing film, and incubate as before for 2 d (*see* **Note 11**).
9 Transfer the explants to shooting medium 2 Leave the plates unsealed and return them to the growth room for a further 2 d (*see* **Note 12**).
10 Reseal the plates with Micropore tape or laboratory sealing film The first regenerating shoots should be visible after 3 wk If the plates become contaminated, *see* **Note 13**

### 3.4. Growth of Transformed Material

1 When the shoots are big enough to handle (approx 1 cm in length), they should be removed from the leaf explant and transferred to rooting medium in 60-mL polypots The shoots usually fall away for the leaf explant very easily, because, by this stage, the explant tissue is brown and dying In contrast, the shoots should be bright green and healthy Only single and well-separated shoots should be removed from each explant, to avoid propagating genetic clones (*see* **Note 14**)
2 Return the shoots to the growth room, making sure that the lids on the polypots are attached loosely, to allow gas exchange Roots should develop within 2–3 wk
3 When the plantlets have three to four pairs of leaves, they should be transferred to either Kilner jars containing MS30 or directly into compost If the plantlet is to be grown in compost, it is important to carefully rinse all the agar from the roots and to autoclave the compost before potting out, to avoid bacterial and fungal growth The plants should be hardened off gradually over a period of 1–2 wk

## 4. Notes

1 Treatment of the seeds with 70% (v/v) ethanol removes waxy substances from the surface of the seeds and kills some contaminating organisms present Do not exceed the immersion time of approx 20 s, because this will result in a loss of viability of the seed
2 Tween-20 acts as a surfactant, to allow access of the sterilizing bleach solution
3 Washing the sterilized seeds five times in $H_2O$ is necessary to remove all of the bleach solution. Failure to do this will result in a loss of viability in a portion of the seed. However, if the germination plates are contaminated with fungi or bacteria and this contamination appears to be associated with the seeds, then either

switch to a fresh batch of seed, or, if this is not possible, extend the sterilization time to 30 min, and reduce the washes to three. Fewer seeds will germinate, but it is worth it if the remaining seedlings are free of any contamination.

4. If there is limited contamination on the plate, then the infected area should be removed as quickly as possible in order to salvage the remaining seedlings.
5. By taking more than one cutting from a shoot-culture, it is very easy to bulk up material rapidly This may be helpful, for example, if tissue is required for DNA or RNA extractions and a plant is also required for potting out, in order to generate seed.
6. If the shoot-culture medium becomes contaminated during the tissue manipulations, it may be possible to rescue the culture by removing the shoot apex and transferring it to fresh medium. However, if the plant material itself is contaminated, it is usually best to dispose of the whole culture by autoclaving There are occasions when it is essential to try to rescue contaminated cultures; this can be attempted in three different ways·
   a. Surface-sterilize the entire plant shoot by immersion, for 10 min, in a solution of 10% (v/v) bleach solution, wash five times in sterile distilled $H_2O$, and transfer to fresh medium This method is only successful if the plant material is contaminated on the surface, rather than systemically
   b Transfer the shoot apex to fresh medium containing 200 mg/L cefotaxime This will inhibit bacterial growth if the shoot is small (<0.5 cm) and is pushed well into the medium
   c Transfer the plantlet (i e , the shoot plus roots) to compost Remove all the agar by rinsing under running $H_2O$, transfer to autoclaved moist compost in a plant pot and cover with plastic film to maintain a high humidity, and allow the plant to harden off gradually over a 2-wk period before transferring to normal greenhouse conditions, allow to flower, then collect seed, from which new shoot cultures can be initiated
7. It is well worthwhile carrying out a rapid plasmid preparation at this stage to ensure that the right plasmid has been transferred to the *Agrobacterium*, and that no major rearrangements have occurred. Bin19 is a low copy-number plasmid, but we have found that starting with a 50-mL culture and using standard miniprep techniques (e.g , **ref.** *7*), enough plasmid DNA is prepared for several restriction enzyme digests
8 Traditionally, the tobacco leaves were cut into disks with a cork-borer, and, although this eliminates the need to remove the midrib and leaf edges (to expose wounded tissue, which is the tissue infected by the *Agrobacterium*), we find that it is much quicker and simpler to cut the leaves into small pieces with a scalpel
9 When culturing the leaf explants with the *Agrobacteria*, it is very important that the bacterial suspension is not too concentrated, because this will lead to an overgrowth of the explant by bacteria, which is very difficult to kill off Therefore the bacterial suspension should be agitated gently (by sucking up and down with a pipet, to break down the clumps of bacteria) and then diluted with fresh LB broth to a final $OD_{600}$ of 1 0

10 Another important step to ensure that the bacteria do not overgrow the leaf explants is to remove excess bacteria by thoroughly blotting the leafs on sterile filter paper prior to transferring them to the shooting medium
11 By sealing the plates with laboratory sealing film, a humid environment is created for the 2 d during which the *Agrobacterium* infection takes place
12 After the infection period, the *Agrobacterium* should be killed off as quickly as possible This can be achieved by embedding the leaf explants well into the medium, and by not sealing the lids on the Petri dishes for a few days After this time, the lids should be sealed with laboratory sealing film again, otherwise, the plates will dry out too rapidly, but this time, cut small slits in the laboratory sealing film to allow gas exchange to occur freely
13 If, while waiting for the transformed shoots to appear, the plates do become overgrown with bacteria, then transfer the leaves to fresh plates The leaves will have expanded considerably and will have become wrinkled, they will therefore have come out of contact with the medium, so, when transferring the tissue, try to embed the edges into the medium
14 Ideally only remove shoots that have regenerated from the edges of the explants that are in good contact with the medium, and, therefore, are kanamycin-resistant It is usual for the edges of the explant to curl away from the medium, thus increasing the opportunity for escapes, but only shoots that are kanamycin-resistant will produce roots in the rooting medium (which contains kanamycin)

## References

1 Horsch, R , Fry, J , Hoffman, N , Eichholtz, D , Rogers, S , and Fraley, R (1985) A simple and general method for transfering genes into plants *Science* **227,** 1229–1231
2 Bevan, M W (1984) Binary *Agrobacterium* vectors for plant transformation *Nucleic Acids Res* **22,** 8711–8721
3 Hoekema, A , Hirsch, P R , Hooykaas, P J J , and Schilperoot, R A (1983) A binary plant vector strategy based on separation of *vir*- and T-region of *Agrobacterium tumefaciens* Ti-plasmid *Nature* **303,** 179,180
4 Figurski, D H. and Helinski, D. R (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans* *Proc Natl Acad Sci USA* **76,** 1648–1652
5 Jackson, M. B , Abbott, A J , Belcher, A R , and Hall, K. C (1987) Gas exchange in plant tissue cultures, in *Advances in the Chemical Manipulation of Plant Tissue Cultures* (Jackson, M B , Mantell, S H., and Blake, J , eds ), British Plant Growth Regulator Group, Bristol, UK, pp 57–71
6 Bullock, W O , Fernandez, J M , and Short, J M (1987) XL1-blue a high efficiency plasmid transforming RecA *E coli* strain with beta-galactosidase selection *Biotechniques* **5,** 376
7 Birnboim, H C (1983) A rapid alkaline extraction method for the isolation of plasmid DNA *Methods Enzymol* **100,** 243–255.

# 39

# Genetic Transformation of Wheat

**Dong-Fang Chen**

## 1. Introduction

Genetic transformation of plants is a technique by which foreign DNA is introduced into plant cells, leading to regeneration of transgenic plants, with new features resulting from integration and expression of the foreign DNA This technique has become an indispensable tool, both for plant biotechnologists to incorporate agronomically useful genes into crops, and for plant molecular biologists to test a particular structural gene or promoter in plant cells. Plant virologists use this technique to study the relationship between a viral pathogen and its host, and to introduce antiviral genes into plants to combat viral diseases.

Wheat (*Triticum aestivam* L ) is the world's largest crop in total production and area. However, despite persistent attempts by numerous laboratories over many years, a technique for successful wheat transformation was reported only very recently. The first transformed wheat plant was reported in 1992 ***(1,2)***; since then, three other groups have obtained fertile transgenic plants, independently ***(3–5)***. There are three common technical features in these reports. First, immature embryos were used as starting materials. This is because wheat immature embryo cultures give rise to a very large number of healthy regenerants within the shortest tissue-culture period. The regeneration is usually initiated from the scutellum tissue through formation of embryogenic callus and somatic embryogenesis. Second, microprojectile bombardment method was used as a means of DNA delivery. With this method, DNA was coated onto very fine heavy metal particles (ca. 1 μm in diameter), which were then accelerated at high speed by compressed helium gas or gunpowder cartridges toward plant cells. Such high-speed particles could penetrate the cell walls and deliver DNA into plant cells. Although such a way of delivering DNA into plant cells

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

appeared to be a very crude method when it was first published in 1987 *(6)*, its potential and effectiveness have become increasingly recognized. For wheat transformation, because there is no versatile biological vector available, and wheat protoplast culture is still an empirical, time-consuming, and genotype-dependent procedure, the microprojectile bombardment of intact tissue, particularly scutellum, became a favorable choice Third, the *bar* gene, which encodes phosphinothricin acetyltransferase (PAT), was used as the selectable marker, and corresponding substrate, L-phosphinothricin (L-PPT), was used as the selective agent. Although this herbicide selection system does not provide a very "tight" selection, it has been shown to be more effective than some antibiotic selection systems tested. The wheat transformation technique described here is a detailed presentation of these three features, supplemented by the author's relevant research experience over many years.

## 2. Materials

1 To maintain a steady supply of plant material, wheat plants (*see* **Note 1**) are grown in a greenhouse or growth chamber at regular intervals, in 6 inch diameter plastic pots filled with potting mixture (Ready Earth from W R Grace or Fisons M2 or F3 compost) Greenhouse and/or growth chamber conditions are as follows the first 40 d at 15°/12°C d/night temperature and 10-h photoperiod (300 $\mu m/m^2/s$), followed by maintenance at 21°/18°C d/night temperature and 16-h photoperiod (300 $\mu m/m^2/s$) Plants are watered every second day and fertilized once a week with 0 4 g/L of soluble greenhouse fertilizer (*see* **Note 2**) Plant spikes are tagged on the first day of anthesis, so that seeds containing immature embryos at correct stage for culture can be collected later.

2 Plasmid DNA, containing a selectable marker (e g , *bar*), a reporter (e g , *gus*A), and genes of interest (e g , a piece of viral genome sequences encoding coat protein [CP] or replicase), is prepared at a concentration of 1 0 µg/µL in sterile $dH_2O$ Good quality DNA (free from RNA and protein contamination) should be used (*see* **Note 3**). Store DNA solution at –20°C

3 The Biolistic PDS-1000/He Particle Delivery System (Bio-Rad), or a biolistic device using gunpowder cartridge is needed (*see* **Note 4**) The biolistic device should be situated in laminar flow hood, and the sample chamber of the device should be kept sterile by spraying 70% ethanol before and after use Rupture disk holder, rupture disks, parts for assembling stopping plate, and sample plate should be sterilized in 70% ethanol and kept under aseptic conditions

4. Tungsten or gold particles, 1.0 µm in diameter, are usually used (*see* **Note 5**). Particles in 60-mg aliquot are washed in 100% ethanol (HPLC or spectrophotometer grade) in a 1 5-mL microcentrifuge tube, then suspended in 1 mL sterile $dH_2O$ A 50-µL aliquot (for 4–8 bombardments) of the particle suspension is pipeted into microcentrifuge tubes (1 5 mL), while vortexing the suspension continually The tungsten particle aliquots should be stored at –20°C to prevent oxidation, gold particle aliquots can be stored at 4°C

5 DNA coating solutions Prepare 2 5*M* $CaCl_2$ and 0 1*M* spermidine (free base) solutions in distilled water Filter-sterilize both solutions Spermine at the same concentration can be used as a substitute for spermidine
6 Media for immature embryo culture are as follows The basic medium is MS basal medium (Imperial) supplemented by 30 g/L sucrose, 100 mg/L inositol (MS0), and 1 or 2 mg/L (MS 1 or MS2) 2,4-dichlorophenoxyacetic acid (2,4-D) For shoot regeneration, MS0 without any phytohormones or with 1 mg/L each of indole acetic acid and zeatin is used *(see* **Note 6**). For root formation, half-strength MS0 medium (1/2 MS) is used The media are adjusted to pH 5 8 using 1*M* HCL or KOH, solidified with 0 25% gelrite, sterilized using autoclaves at 120°C for 20 min, and are poured into 9-cm Petri dishes or Magenta cultural vessels For selection, add 5 mL or 10 mL L-PPT stock solution (*see* **Subheading 3.8.**) into MS0 or 1/2 MS media, mix well, and pour them into 9-cm Petri dishes or Magenta culture vessels
7 β-Glucuronidase (GUS) histochemical assay solution 0 5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (x-gluc) Dissolve 25 mg x-gluc in 3 mL dimethy sulfoxide (DMSO) in a 100-mL beaker, add 50 m*M* of pH 7 0 phosphate buffer with 0 01% Triton X-100, to a final volume to 50 mL Filter-sterilize the solution and store at –20°C
8. Selection stock solution 1 mg/mL, L-phosphinothricin (L-PPT) stock solution in $dH_2O$ (*see* **Note 7**) Filter-sterilize the stock solution and store at –20°C

## 3. Methods

### 3.1. Preparing Wheat Immature Embryos

1 Collect immature seeds 12–14 d after anthesis and surface-sterilize the seeds using 10% Domestos for 20 min, followed by thorough rinsing with sterile water
2 Dissect the seeds to excise immature embryos, using fine needles under a low-power (magnification ×16) stereo microscope placed in a laminar flow hood Immature embryos at this stage are about 1.0–1.2 mm long and semitransparent (*see* **Note 8**)
3. Place the immature embryos on the MS2 medium, with the axis in contact with medium (the scutellum facing upward), 20–30 embryos as a cluster in the middle of a Petri dish (*see* **Note 9**)
4. Preculture the embryos at 25°C in darkness for 2 d before they are subjected to bombardment

### 3.2. DNA Coating and Loading

1 To an aliquot of 50 µL of the particle solution in a 1 5-mL microcentrifuge tube, add, in order, under continuous vortexing, 5 µL of DNA, 50 µL of $CaCl_2$, and 20 µL of spermidine, and continue vortexing for 5 min
2 Spin the microcarriers (the DNA-coated particles) in a microcentrifuge for 20 s; remove and discard as much supernatant as possible
3 Wash the microcarriers with 250 µL of 100% ethanol (HPLC or spectrophotometer grade) by vortexing briefly, and centrifuge for 20 s, remove and dis-

card the supernatant; resuspend the microcarriers in 60 µL of 100% ethanol (*see* **Note 10a**)

4. Pipet 6 µL of the microcarrier suspension onto the center of the macrocarrier already installed in the macrocanier holder
5. Let microcarrier/macrocarrier dry in a low-humidity and vibration-free environment for about 1 min To obtain the best results, use the prepared macrocarriers as soon as possible *(see* **Note 10b**)

### 3.3. Microprojectile Bombardment

**Caution:** Safety glasses should be worn by everyone in the area when the PDS-1000/He or a different biolistic device is being operated. The procedures described below are for the use of the PDS-1000/He.

1 Set the helium pressure at 1300 psi (or another pressure 200 psi above the burst pressure of the selected rupture disk) by turning the regulator adjusting-screw clockwise
2 Place an 1100-psi rupture disk in the rupture disk retaining cap, screw, and tighten the cap onto the gas acceleration tube (*see* **Note 11**)
3 Place a sterile stopping screen on the stopping screen support (*see* **Note 12**) Install the macrocarrier holder with macrocarrier on the top rim of the fixed nest The microcarriers should be facing down toward the stopping screen Place the macrocarrier cover lid on the assembly and turn clockwise until snug Place the macrocarrier launch assembly in the second slot from the top in the sample chamber
4 Place the precultured wheat embryos (in a Petri dish without the lid) at a distance of 13 cm from the stopping plate and close the sample chamber door tightly
5 Turn the vacuum pump on Set the VACUUM switch on the PDS-1000/He to the VAC position to evacuate the sample chamber to 26–28 in of mercury Put the vacuum switch in the HOLD position.
6. Press and hold the FIRE switch until the rupture disk ruptures (*see* **Note 13**), release the FIRE switch immediately after the disk ruptures
7 Release the vacuum in the sample chamber by setting the VACUUM switch to the VENT position
8. Open the chamber door, remove and cover the Petri dish
9 Reassemble rupture disk and microcarrier launch assembly for next bombardment (*see* **Note 14**)

### 3.4. Recovery of Bombarded Embryos and Assessment of DNA Delivery

1 Rearrange the treated embryos by placing 10 of them in a Petri dish containing fresh MS2 medium, grow them in darkness at 25°C for 2 d
2 If the *gus*A gene is used as a reporter (*see* **Note 15a**), take a few embryos for GUS histochemical staining using the x-gluc solution *(7)*. Incubate the embryos in the x-gluc solution for 12–18 h at 37°C, then transfer the embryos into 70% ethanol for fixation As a result of delivery and expression of the *gus*A gene, blue spots

(singles cells or cell clusters) will appear on the scutellum tissue of the embryos after staining Record the number and distribution of the blue spots (per embryo or per bombardment) to obtain transient gene expression frequency data Such data provide information on the effectiveness of the DNA delivery (*see* **Note 15b**)

3. Continue to culture the rest of the bombarded embryos at 25°C in darkness for another 2 wk

### 3.5. Selection and Regeneration of Transformed Shoots

1 After 2–3 wk culture, callus will form from the scutellum tissue of the immature embryos Transfer these embryo–callus cultures to MS1 medium containing 5 mg/L L-PPT in 9-cm Petri dishes and place the cultures at 25°C under fluorescent light (3000 lux for 16 h). Presence of L-PPT in the MS1 medium allows transformed cells and cell clusters to proliferate, while suppressing the growth of the untransformed cells (*see* **Note 16**)

2. Allow these embryo–callus cultures to grow for another 2–3 wk, somatic embryo structures and green spots will appear on some of the calli. Transfer these calli to MS0 medium containing 5 mg/L L-PPT in 9-cm Petri dishes for shoot development, leave the rest of the calli to continue to grow until they form morphogenic structures

### 3.6. Selection and Regeneration of Plantlets

1. After a further 2–4 wk, shoots will form on the L-PPT containing MS0 medium Shoots normally grow in clumps. When their leaves reach 1–2 cm, dissect the shoot clumps into individual shoots, transfer them into 1/2 MS medium containing 10 mg/L L-PPT in Magenta culture vessels (4–5 shoots into a box), and culture them under the same growth condition

2 Truly transformed shoots will produce healthy roots at the base of the shoots, to form the transgenic plantlets ($T_0$ plantlets). Allow the $T_0$ plantlets to grow until their leaves reach to the lid of the Magenta culture vessel A small proportion of untransformed shoots, which escaped from the first two rounds of selection, will have bleached leaves and will not form healthy root system (*see* **Note 17**)

3 For winter varieties, the $T_0$ plantlets can be vernalized at 4°C for 4–8 wk at this stage

### 3.7. Growth of $T_0$ Plants Into Maturity

1 Gently pull the plantlets out of the Magenta culture vessel. With great care, wash away with tap water the Gelrite or agarose surrounding the roots, so that the root system is not damaged

2. Transfer the plantlets into pots containing Fisons F3 (Fisons) compost. It is useful to provide these plantlets with a low-humidity condition (about 30–50%) in a growth chamber for 1–2 wk, then transfer them into a contained greenhouse

3 Maintain these plants in the greenhouse condition as described above (*see* **Subheading 2., item 1**) Cover all of the spikes of the $T_0$ transgenic plants with crossing bags during the flowering stage (*see* **Note 18**)

4. For the maturation of the seeds of the $T_0$ plants ($T_1$ seeds), cease to water, and feed the plants 30 d after the pollination
5. Harvest and label the $T_1$ seeds from each individual spike
6. Autoclave the rest of naturally dried plant parts (straw, old leaves, and roots) and compost for disposal

### 3.8. Phenotypic, Genetic, and Molecular Analyses of Transgenic Plants and Their Progenies

1. For phenotypic and genetic analyses, germinate the $T_1$ seeds (and some untransformed seeds as negative control) in 9-cm Petri dishes containing L-PPT solution (10 mg/L, soaked in filter paper) The seeds that have the functional *bar* gene will germinate and produce normal seedlings, but the seeds that do not have the *bar* gene will produce stunted and bleached leaves without a healthy root system
2. Collect information about the germination frequency based on seeds from individual spikes If a single *Locus* integration of the *bar* gene had occurred in the $T_0$ plants, a typical Mendelian segregation of 3·1 (resistant·sensitive) would be observed
3. An alternative test is to germinate the $T_1$ seeds in soil and grow the seedlings for 3–5 wk, then spray the seedlings with 2% Basta. The sensitive plants will show bleached leaves a few days after the spraying and subsequently be killed, but the resistant plants will grow normally The ratio of resistant to sensitive plants would be 3 1, if transgenes are integrated at a single *Locus* (*see* **Note 19**)
4. Choose L-PPT or Basta resistance plants, collect plant parts at different stages of development, e g , leaf, root, ovary, anther, and microspore, for GUS assays (*see* **Subheading 3.4.2.**)
5. Detection of PAT enzyme activity can also be conducted ***(8)*** in the L-PPT-resistant $T_0$ and $T_1$ plants.
6. Southern hybridization and polymerase chain reaction (PCR) analyses to confirm stable integration and desired expression of the transgenes (*see* **Note 20** and Chapters 41–43).
7. Northern hybridization analysis to detect the transcripts of the transgenes (*see* Chapter 44)
8. To detect the product of a particular transgene, which is not readily detectable using enzymatic assays, e g , viral CP or replicase, the Western hybridization analysis is necessary (*see* Chapter 45)

## 4. Notes

1. The choice of wheat variety for the transfommation test is important. So far, only those varieties that are highly regenerable under tissue culture conditions, are suitable for transformation tests. The varieties that are shown to be amenable to transformation include the following varieties Bob White, Pavon, RH770019, Florida, and Fielder If transformation targets are commercial varieties, it is recommended that a regeneration test is carried out in advance

Winter wheat varieties require vernalization treatment at a young seedling stage (4–28 d after germination). The wheat seedlings are placed in cold room or refrigerator at 4°C for 6–8 wk before they are transferred to a greenhouse or growth chamber.

2 To maintain wheat plants free from pests and diseases, spraying insecticide and fungicide is sometimes necessary.

3 Contamination by RNA and/or proteins in DNA samples affects the quality of DNA coating onto the particles RNase (DNase-free) and protease K can be used to remove RNA and protein contamination, respectively It is recommended to check the plasmid DNA's purity using agarose gel electrophoresis before coating The form of DNA can be either supercoiled or linear

4 If a gunpowder cartridge device is used, installation of a nylon membrane (250-μm meshes) between the stopping plate and the sample plate in the sample chamber is recommended This could reduce the cell damage caused by the shock wave and gunpowder residues.

5 The choice of particles (gold or tungsten) is a matter of personal preference No difference at a transient gene expression level was observed after comparison of the two types of particles for DNA delivery into wheat and other cereal cells (Chen and Dale, unpublished data) Electron-microscopic examination showed that gold particles were spherical and uniform, while tungsten particles were less unifomm in size and polygon in shape. Gold particles, however, are about 120 times more expensive than tungsten particles (Bio-Rad)

6. Although most wheat varieties respond to an order of culture on MS2, MS1, and MS0 media by regeneration through somatic embryogenesis, some varieties may require special media for a higher frequency of regeneration. The L3 medium *(4)* has been used to promote regeneration.

7. L-phosphinothricin (Sherman) is the active ingredient of the herbicide Basta Basta can also be used as a selective agent Proper conversion to obtain the correct concentration of an active ingredient is needed when using Basta

8 Seeds at different positions along the spike mature at a different rate Seeds at the central part of the spike normally mature earlier than those at the temminal positions. Therefore, immature embryos harvested 12–14 d after anthesis often show different sizes and colors, which represent different degrees of maturity. Embryos longer than 13 mm, with opaque white color, are too old to be cultured (they will germinate on medium), and embryos less than 10 mm, with total transparency color, are too young to be cultured.

9 Because of the low transformation frequency when using microprojectile bombardment, 600–1000 immature embryos are usually needed to produce one or a few transgenic plants for testing one DNA construct

10 a. Coating of DNA onto the particles occurs more evenly if the preparation is kept under continuous agitation. This can be achieved by using a multiple-placement platform attachment head on a vortex mixer Once $CaCl_2$ is added to the mixture, particle-DNA aggregates will form. Continuous vortexing at this stage, and further vortexing after adding spermidine, are very important. After adding

ethanol to the microcarriers, the aggregates should become dispersed again If large aggregates persist, it may mean that either DNA was not sufficiently clean or DNA concentration is too high Presence of the microcarrier aggregates affects the evenness of loading the microcarriers onto macrocarriers

b To obtain uniform amounts of microcarriers, all pipeting should be done from a continuously vortexed tube. Pipeting should be done rapidly, because microcarriers settle out quickly, even in the pipet tip Dispense the microcarriers onto the macrocarriers and allow them to dry only in a vibrationless environment, because vibration enhances the agglomeration of microcarriers High humidity results in rapid water absorption by the ethanol, resulting in slower drying This can lead to serious agglomeration of the microcarriers

11 If the retaining cap is not tightened sufficiently, the rupture disk may slip out of place before it ruptures This will not properly launch the macrocarrier to accelerate the microcarriers The rupture disks should not be handled with bare hands at any stage, because the grease and sweat left on the disk prevent it from holding its position properly, even when the cap is tightened To remove grease or dust, the disk can be soaked in isopropanol or pure ethanol immediately before use

12 Occasionally, the operator forgets to put the stopping screen in the stopping screen support. When the rupture disk bursts, this will cause the macrocarrier to pass straight through the stopping plate, which destroys the sample Therefore, always double-check the placement of the stopping screen

13 A metering valve is installed in the solenoid valve assembly to control the rate of fill of the gas acceleration tube It should take about 12–15 s to fill to bursting pressure The gage at the top of the acceleration tube should be observed A more rapid fill rate may result in what appears to be a lower bursting pressure, because of gage lag The metering valve is preset, but may be adjusted, if desired.

14 If necessary (e g , to test transient gene expression), multiple bombardments can be applied to the same sample More DNA particles will be delivered to the target tissues, but cell damage caused by more particles, multiple shock wave, and vacuum, is more severe Control samples should also be set up at this stage, i e immature embryos bombarded with particles coated with the calf thymus DNA

15 a Although *gus*A gene is the most widely used reporter gene for plant transformation test, maize *C1/Lc* genes ***(9,10)*** can also serve as reporter for wheat transformation ***(11)*** The *C1/Lc* genes encode *trans*-factors, which regulate anthocyanin biosynthesis in maize. Delivery and expression of the *C1/Lc* genes into wheat cells results in cell-autonomous coloration (in most cases, red color) The advantages of using such a reporter system are. Gene expression can be easily visualized on target tissues and cells without using a destructive assay, cell-autonomous coloration allows more accurate counting of the gene expression events, it represents a more accurate estimation of DNA delivery events than the *gus*A gene in wheat. But the disadvantage of using this reporter system is that it can only be used as a marker for transient gene expression, because these regulatory genes could impinge upon other cellular and physiological processes This requires the *C1/Lc* genes to be coated on particles separately from the target genes

b If no or few blue spots were observed after x-gluc staining, it may mean that DNA coating or bombardment parameters (rupture pressure, distance between the sample and the stopping plate, and the macrocarrier travel distance, and so on) were not optimal

16 Imposition of selection can be earlier or later than the time specified here The earliest selection may be applied 2 d after bombardment The late selection may be applied at the plantlet stage, which is about 6–8 wk after bombardment The early selection normally provides a tight procedure to catch the transformants, but it may reduce regeneration frequency as a result of suppressing the proliferation of the majority of the untransformed cells The late selection gives all the cultured tissues a maximum opportunity to regenerate, but unavoidably allows some untransformed shoots to escape the selection

17 Observation of root formation from the preselected shoots on the 1/2 MS medium with 10 mg/L of L-PPT helps to recognize the true $T_0$ plantlets The roots of the real transformants will grow into the medium and develop lateral roots at the bottom of the culture vessel; the escapes or chimeric transformants will have poor initiation of roots, which cease to grow when the root tips touch to the surface of the selection medium At this stage, small sections of leaves or roots from the plantlets can be collected for GUS histological assay, which will provide further information about the stable transformation status

18. Although wheat is an inbred species, to avoid possible genetical complication in the transgenic progeny resulting from crosspollination between individual transgenic plants, it is necessary to bag the flowering spikes

19 Further genetical analysis of the $T_1$ plants by crossing them with the wild-type plants is sometimes necessary This test is particularly useful in obtaining genetic information from those $T_1$ plants that have multiple copies of the transgene integrated into different positions on the nuclear genome

20. In the Southern hybridization analysis (*see* Chapters 41 and 43) of transformed plants derived from the direct DNA delivery, it is often observed that common rearrangement patterns of transgenes obscures the hybridization pattern of the integration The *Dpn*1-aid Southern hybridization technique ***(12)*** may be used to remove all the possible $N^6$-methyladenine DNA

## References

1 Vasil, V, Castillo, A M, Fromm, M E, and Vasil, I K (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus *BioTechnology* **10,** 667–647.

2 Vasil, V, Srivastava, V, Castillo, A M, Fromm, M E, and Vasil, I K (1993) Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos *BioTechnology* **11,** 1553–1558.

3 Weeks, J T, Anderson, O D., and Blechl, A. E. (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*) *Plant Physiol* **102,** 1077–1084

4 Becker, D , Brettschneider, R , and Lorz, H (1994) Fertile transgenic wheat from microprojectile bombardment of scutella tissue *Plant J* **5,** 299–307
5 Nehra, N S , Chibbar, R N , Leung, N , Caswell, K , Mallard, C , Steinhauer, L , Baga, M., and Kartha, K K. (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs *Plant J* **5,** 285–297
6 Klein, T. M , Wolf, E D , Wu, R , and Sanford, J C (1987) High-velocity microprojectiles for delivering nucleic acids into living cells *Nature* **237,** 70–73
7 Jefferson, R. A. (1987) Assaying chimeric genes in plants· the GUS gene fusion system *Plant Mol Biol Rep* **5,** 387–405
8 Spencer, T M , Gordon-Kamm, W., Daines, R J , Start, W G., and Lemaux, P G (1990) Bialaphos selection of stable transformants from maize cell culture *Theor Appl Genet* **79,** 625–631
9 Goff, S A , Klein, T M , Roth, B A , Fromm, M E , Cone, K C , Radicella, J P , and Chandler, V L. (1990) Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues *EMBO J* **9,** 2517–2522
10 Ludwig, S , Bowen, B , Beach, L , and Wessler, S. (1990) A regulatory gene as a novel visible marker for maize transformation *Science* **247,** 449,450
11 McCormac, A C , Wu, H , Bao, M , Wang, Y -B , Xu, R , Elliott, M C , and Chen, D F (1997) The use of visual markers as cell-specific reporters of *Argobacterium*-mediated T-DNA delivery to wheat (*Triticum aestivum* L ) and barley (*Hordeum vulgare* L ). *Euphytica*, in press
12 Chen, D F., Dale, P J , Heslop-Harrison, J S , Snape, J W , Harwood, W , Bean, S , and Mullineaux, P M (1994) Stability oftransgenes and presence of $N^6$ methyladenine DNA in transformed wheat cells *Plant J* **5,** 429–436

# 40

# Production of Transgenic Rice (*Oryza sativa* subspecies *japonica* cv. Taipei 309)

**Wallace G. Buchholz, Weimin Teng, Delphia Wallace, John R. Ambler, and Timothy C. Hall**

## 1. Introduction

*Oryza sativa* L. has three subspecies: *indica*, *japonica*, and *javanica* Subsp *indica* grows well in southern temperate and tropical regions; subsp. *japonica* grows well in cooler climates such as Japan; and subsp. *javanica* is grown mostly in the Americas and Europe. Several viruses infect rice, causing devastating losses in yield. Tungro disease is caused by an association of an RNA genome virus (rice tungro spherical virus) and a DNA genome virus (rice tungro bacilliform virus). Estimated annual yield losses caused by Tungro virus infection of rice exceed $1.5 billion *(1)*. Rice ragged stunt virus causes the second most important viral disease, with economic losses exceeding $140 million annually. Several tenuiviruses are also major pathogens in various rice-growing regions. These include rice grassy stunt virus, which is prevalent in the Philippines, rice stripe virus, which is often found in Japan; and rice hoja blanca virus, which is endemic in Latin America and occurs in sporadic but disastrous outbreaks. Novel biotechnological approaches for resistance, using various pathogen-derived genes, are being explored *(2)*. The production and thorough molecular analysis of transgenic plants by methods such as those described in this chapter and in Chapter 41 are vital toward evaluation of the efficacy of these new approaches.

The method presented in this chapter is primarily for transformation of *O sativa* subsp. *japonica* cv Taipei 309 (T309) by particle bombardment *(3)* using the commercially available helium-driven PDS1000/He system (E. I du Pont de Nemours, Wilmington, DE). Discussions of the biolistics process and optimization of conditions have been presented *(4,5)*, and an excellent monograph of rice anatomy has been published *(6)*.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

A major advantage of the biolistics system is that it is useful for a wider range of cultivars than other systems currently available for stable transformation of rice. For instance, transformation and regeneration have been successful using a subsp. *javanica* cultivar, Gulfmont (W Teng, W. G. Buchholz, and T. C. Hall, unpublished), and a subsp. *indica* cultivar, TN1 *(7)*, by the biolistics approach. Although it is less genotype-dependent than other available methods, biolistics is still limited to those cultivars that are amenable to regeneration from tissue-culture. The major disadvantage of the biolistics system is the same as that for all other direct DNA uptake systems; fragmentation and rearrangement of input DNA leads to insertion of functionally intact genes of interest at relatively low frequencies (approx 1–2% of transformants).

Two other systems have been developed for stable transformation of rice· direct DNA uptake by protoplasts and *Agrobacterium*-mediated transformation Protoplast transformation has received much attention and depends on physical (e.g., electroporation *[8,9]*) or chemical (e.g., polyethylene glycol [PEG] *[10]*) means to stimulate DNA uptake. Electroporation-mediated transformation, with which we have many years experience *(11)*, can be an efficient way to produce large numbers of independently transformed plants with relatively low numbers of copies of the genes of interest. Similarly, PEG-mediated transformation produces many transformants, but, in our hands, the number of copies of the introduced genes tends to be very high. The primary disadvantages to protoplast systems are that they are currently limited to only a few cultivars, they are labor intensive, and the embryogenic cultures are extremely sensitive to environmental fluctuations and equipment failures. Also, depending on the culture and its age, the frequencies of cell-wall regeneration from protoplasts, plant regeneration from calli, and fertility vary widely from one experiment to the next

Though highly sought after, *Agrobacterium*-mediated transformation of rice was not convincingly shown until recently *(12)*. Using the same bacterial strains and plasmids as that study, we have successfully repeated transformation of rice callus tissue and have regenerated transgenic plants *(13)* This is an exciting development, because of the very desirable characteristic of the *Agrobacterium* system in which a discrete portion of DNA is integrated into the genome. This yields a high frequency of cotransformation of an intact copy of the gene of interest with the selectable marker. It remains to be seen whether the *Agrobacterium* system will be highly genotype dependent.

The protocol presented below, although workable and currently producing transgenic rice plants from approx 10% of the bombarded embryos, should be taken as a starting point. As with any methodology, this protocol continues to evolve as more experience is gained and as more parameters are varied and

optimized. Using the stated conditions, the approximate time from isolation of embryos to having transgenic seed from Taipei 309 varies from 6 to 9 mo.

## 2. Materials

### 2.1. Embryo Isolation

1 Single-edge razor blades
2 Bleach (50%), prepared by diluting commercial bleach (5 25% HClO) with an equal volume of sterile water just prior to use Add two drops of Tween-20 per 100 mL
3 Sterile jewelers forceps (e g , cat no. 4380, Hamilton Bell, Montvale, NJ).
4 Betadine (10% Iodine antiseptic solution)
5 Sterile 100 × 15-mm culture plates.
6 Sterile 100 × 25-mm culture plates
7 Ethanol (EtOH, 70%) in a spray bottle.
8 LS 2 5 tissue-culture medium *(14)*
   a To 990 mL ultrapure $H_2O$, add the following, with stirring 1 package Murashige & Skoog Salt Mixture (*[15]*, cat. no 11117-066, Gibco-BRL, Gaithersburg, MD), 30 g sucrose, 100 mg myo-inositol, 0 4 mg thiamine-HCl (0 4 mL of a 1-mg/mL solution in $H_2O$), 2 5 mL of a 1-mg/mL 2,4-D solution (2,4-dichlorophenoxyacetic acid, cat no D 8407, Sigma, St Louis, MO, dissolved in 0 02*M* NaOH or 95% EtOH) Adjust pH to 5 8 with 0 5*M* KOH
   b Add 3 85 g agarose (Sigma Type I, cat no A6013) per 1-L media bottle, and add medium
   c Leave bottle lid loose, cover lid and neck with aluminum foil, and autoclave (121°C, 25 min, liquid cycle) After autoclaving, swirl medium to evenly distribute agarose and cool to 50°C.
   d. Transfer bottles to a laminar flow hood, spray, and wipe with 70% EtOH
   e If needed, add filter sterilized selective agent (e g , 4 mg bialaphos/L; *see* **Subheading 2.3.**)
   f. Pour approx 20–25 mL per 100 × 15-mm plate used for embryo isolation and bombardment For selection plates, pour approx 50 mL per 100 × 25-mm plate
   g Label plates and leave overnight in a laminar flow hood (with fan off) to dry

### 2.2. Biolistics (Four Plates of Embryos, Four Shots Per Plate)

1 Bio-Rad helium-driven PDS1000/He system
2 Grade 5 helium gas in a cylinder with at least 1500 psi
3 250 mL 70% EtOH, prepared just prior to use
4 100 × 25-mm sterile tissue-culture plates
5 Eight 1100-psi burst disks (Bio-Rad)
6 Eight 1300-psi burst disks (Bio-Rad)
7 Sixteen macrocarrier holders (Bio-Rad)
8 Sixteen macrocarriers (Bio-Rad)
9 Sixteen stop screens (Bio-Rad)

10 Macrocarrier loader (Bio-Rad)
11 Box of laboratory wipes (Kimwipes) wrapped in aluminum foil and autoclaved
12 Gold microcarriers (1.0 μm) (Bio-Rad)
13 Plasmid DNA encoding a chimeric phosphinothricin acetyl transferase gene *(16)* (e g , we have used either the CaMV 35S [Huntley and Hall, in preparation] or maize ubiquitin 1 [Christensen and Quail, in preparation] promoter, coupled with the nopaline synthase polyadenylation signal, to drive expression in rice)
14 Plasmid DNA encoding the gene of interest to be introduced
15 0 1*M* Spermidine Dissolve the free base in water, filter-sterilize, and store at –20°C Discard after 1–2 mo
16 $CaCl_2$ (2 5*M*), filter-sterilize, and store at –20°C, stable for months
17 50% Glycerol, filter-sterilize, make up as needed

## 2.3. Selection and Regeneration

1 LS 2 5 tissue-culture medium supplemented with 4 mg/L bialaphos.
2 MSD4 tissue-culture medium
   a To 985 mL ultrapure $H_2O$, add the following with stirring 1 package Murashige and Skoog Salt Mixture (*[15]*, Gibco-BRL, cat no 11117-066), 30 g sucrose, 100 mg myo-inositol, 1 mL of MS vitamins + glycine (1000 X stock, *see below*), 0 5 mL BAP (6-benzylaminopurine, Sigma cat no B 9395, 1 mg/mL stock in 0 1*M* HCl), 50 μL NAA (naphthalene acetic acid, Sigma cat. no. N 0640, 1 mg/mL stock in EtOH). Adjust pH to 5 80 with 0 5*M* KOH Add 3 85 g agarose (Sigma Type I, cat no A6013) per 1-L media bottle, and add medium. Cover bottle lid and neck with foil, leave lid loose, and autoclave (121°C, 25 min) After autoclaving, swirl medium to evenly distribute agarose and cool to 50°C Transfer bottles to a laminar flow hood, spray, and wipe with 70% EtOH Pour approx 50 mL per 100 × 25-mm plate Leave plates overnight in laminar flow hood (with fan turned off) to dry
   b MS vitamins + glycine (1000X stock, store at –20°C, stable for months). To 50 mL ultrapure $H_2O$, add the following, with stirring 25 mg nicotinic acid, 25 mg pyridoxine HCl, 5 mg thiamine-HCl, 100 mg glycine.
3 MS0 medium: Prepare as for MSD4, except omit BAP and NAA After adding the agarose and medium to the media bottle, fully dissolve the agarose by heating (Phytagel, Sigma cat no P 8169 may be substituted for agarose) Aliquot 60–65 mL per Magenta box (Sigma cat no. V8505) and put the box lids on Autoclave (121°C, 25 min) and transfer Magenta boxes directly to a laminar flow hood to cool. Stacking boxes helps eliminate condensation on the lids

## 2.4. Plant Growth

1. Shredded pasteurized peat moss
2 Coarse vermiculite
3. 1-gal Plastic pots
4 12 × 2-in Round trays
5 1 × 2-mm Mesh nylon screen

**Table 1**
**Mikkelson's Nutrient Solution[a]**

| Constituent | Stock solution, g/L | Nutrient solution, mL stock solution/L |
|---|---|---|
| $KNO_3$ | 101 1 | 6 0 |
| $Ca(NO_3)_2$ $4H_2O$ | 236 2 | 4 0 |
| $NH_4H_2PO_4$ | 115.1 | 2.0 |
| $MgSO_4 \cdot 7H_2O$ | 246 5 | 1 0 |
| Micronutrient stock solution | [b] | 1 0 |
| Iron cheleate 330 | [c] | 0 2 g |

[a]Make a separate stock solution of micronutrients and each macronutrient Dilute stock solutions to prepare final nutrient solution

[b]For micronutrient stock solution, dissolve the following in 1 L water while mixing 3 728 g KCl, 1 546 g $H_3BO_3$, 0 338 g $MnSO_4$ $4H_2O$, 0 575 g $ZnSO_4$, 0 125 g $CuSO_4$ $5H_2O$, 0 081 g $H_2MoO_4$ (85% $MoO_3$)

[c]Add iron cheleate as a solid to the final nutrient solution

6. Reverse osmosis purified or deionized water.
7 1-gal Plastic bags (e g , Ziplock freezer bags, Dow, ~11 × 11 in , 2 7 mil)
8 Mikkelson's *(17)* nutrient solution (*see* **Table 1**)

## 3. Methods

### *3.1. Preparation of Embryos*

1 Collect panicles 12 d after flowering and keep the cut ends submerged in water until use Choose seeds that are almost fully formed and are close to the end of the milky stage The embryos should be approx 2.0 mm long (1.7–2 2 mm) (*see* **Note 1**)
2. On a clean area, but not necessarily in a laminar flow hood, remove the spikelet from the panicle by cutting through the rachilla at the base of the grain. Be especially careful to cut high enough to facilitate removal of the lemma and palea, but low enough to avoid damaging the embryo
3 Using a jewelers forceps, peal away the lemma and then the palea, being careful not to damage the pericarp (which should be light green) on the surface of the grain This can be difficult, since the lemma and palea are interlocked and do not separate easily
4. The dehulled seeds should be immediately submerged in sterile water and left there while subsequent seeds are being processed
5. Transfer the dehulled seeds to the bleach solution (100 mL for each 100–150 seeds) in a flask and vacuum infiltrate (~1 mmHg) for 5–10 min, gently swirling each 1–2 min Adequate vacuum is being applied if all the seeds initially float and the surface of the liquid is covered with fine bubbles By the end of the vacuum treatment, many of the seeds should sink and the bubbles on the surface will be much larger.

6 After the vacuum treatment, leave seeds in the bleach for another 15–20 min, swirling occasionally By the end of the treatment, most of the green will have been bleached from the pericarp
7 Transfer the flask to a tissue-culture hood; spray the flask and all materials introduced into the hood with 70% EtOH Wash hands and forearms with Betadine and use aseptic technique for subsequent manipulations
8 Decant the bleach, discarding any seeds that float (these tend to have undersize embryos), and distribute 75–100 seeds per 100 × 25-mm tissue-culture dish Add 25–30 mL sterile water to each culture dish and gently shake until all the seeds are submerged Thoroughly decant the water and repeat this wash for a total of at least three washes
9 Transfer approximately five seeds to a sterile 100 × 15-mm tissue-culture dish lid or bottom
10 Hold a seed with the tips of one forceps and use the other to carefully peal the pericarp from the embryo end of the seed Puncture the seed coat (testa and exosperm layers) and dislodge the embryo from the endosperm.
11 Place the embryo on LS2 5 medium so that the flat side (plumule and radicle side) is in contact with the medium and the scutellum is up
12 Repeat **steps 9–11**, arranging 40–60 embryos in a rectangular pattern centered in the dish
13. Incubate overnight at 26°C in the dark
14. Examine for contamination and, if present, subculture axenic embryos onto fresh plates Be careful not to mistake endosperm starch granules for contaminants

### 3.2. Preparation for Biolistics

It is assumed anyone using a PDS1000/He biolistics system for bombardment of rice tissues has access to the Bio-Rad operation manual. Therefore, details of the basic operation of the system will not be covered here. Rather, the following protocol will only discuss details and variables, as needed, to clarify the rice transformation process.

All the following steps (until the plants are put into soil) must be done with aseptic technique in a laminar flow or biocontainment hood It cannot be overemphasized that one of the primary reasons for failure of an experiment is contamination.

Preparation of microcarriers can be done several days in advance and, as indicated above, embryo isolation should be done 1–2 d in advance, in order to detect and minimize contamination. The microcarriers should not be coated with DNA until just prior to bombardment

1. Thoroughly spray the biolistics apparatus and the interior of the hood with 70% EtOH. If possible, also irradiate the contents of the hood with a germicidal lamp
2. Distribute the burst disks, stop screens, macrocarrier holders, macrocarriers, and macrocarrier loader into 100 × 25-mm culture dishes and cover them with 70% EtOH

3 Allow all components, except the burst disks, to soak for at least 1 h to sterilize Sterilize the burst disks by soaking for 5 min. After sterilization, transfer all components to a layer of autoclaved wipes to dry

### 3.2.1. Preparation of Microcarriers

The following method is modified slightly from that of Heiser *(5)* and was originally developed by Sanford et al. *(4)*. It allows the preparation of 30 mg of particles, which would be adequate for 50 individual bombardments. We usually prepare 30 mg, since the particles can be stored for only about 3 wk at 4°C, or 2 wk at room temperature. For ease of handling, when 60 mg are needed, we prepare two tubes with 30 mg each.

1 In a 1 5-mL microcentrifuge tube, vigorously vortex 30 mg of particles in 0 5 mL freshly prepared 70% EtOH for 5 min
2 Incubate particles at room temperature for 15 min
3 Microcentrifuge (15,000*g*) for 5 s and decant
4 Wash particles three times as follows
   a Add 0.5 mL sterile water
   b. Vortex for 1 min
   c Allow particles to settle for 1 min
   d Microcentrifuge for 5 s and decant
5 Add sterile 50% glycerol to bring the particle concentration to 60 mg/mL
6 While vigorously vortexing, distribute 40-µL aliquots of particles into 1 5-mL microcentrifuge tubes

### 3.2.2. Coating DNA on Microcarriers

1 Add 1 and 3 µL (1 mg/mL) $CsCl_2$-purified plasmid encoding the bialaphos-resistance gene and the gene of interest, respectively, to a 40 µL aliquot of particles (*see* **Note 2**). We usually bombard with four different constructs in a given experiment, which results in coating four aliquots of particles at a time
2 Place tubes on a table mixer (e g , Eppendorf Model 5432) and mix for 5 min
3 While vortexing vigorously, add 40 µL $CaCl_2$ (2.5*M*) and then 16 µL spermidine (0 1*M*)
4. Continue vigorous vortexing for 3 min
5 Allow particles to settle (for at least 1 min) while subsequent aliquots are being processed.
6 Microcentrifuge for 4–5 s
7 Draw off supernatant with a pipet and discard
8 Without disturbing pellet, add 112 µL freshly prepared 70% EtOH
9 Draw off supernatant with a pipet and discard
10. Again without disturbing pellet, add 112 µL 100% EtOH
11. Draw off supernatant with a pipet and discard
12 Suspend particles in 39 µL of 100% EtOH (a few microliters will be lost to evaporation and 36 µL are needed) by mixing with the pipet tip and then vigorously pipeting up and down

### 3.2 3. Loading Macrocarriers

1 For each construct to be bombarded, insert four sterile macrocarriers into macrocarrier holders, using the macrocarrier loader Rotating the loader 90–180 degrees, while pressing down, helps in seating the macrocarrier Place each set of 4 into a labeled sterile culture dish
2 Place a set of four macrocarriers to be loaded with particles on sterile lab wipes in the rear of a laminar flow hood
3. With the DNA-coated particles evenly suspended by vigorous vortexing or pipeting, remove a 9-μL aliquot and quickly spread it over the central portion of a macrocarrier, i e , the area directly above the opening in the holder The ethanol will quickly evaporate (*see* **Note 3**)
4 Repeat **step 3**, loading the other three macrocarriers with the particles coated with the same construct Once the ethanol has evaporated from the last macrocarrier, place all four back into the labeled culture dish
5 Repeat **steps 2–4** for particles coated with each of the other constructs

### 3.2.4. Particle Gun Parameters

1 Rupture disk to macrocarrier gap one-eighth in
2 Macrocarrier travel distance 6 mm
3 Target distance approx 11 cm, i e , target platform in second slot from bottom
4 Helium pressure· We routinely bombard each set of embryos four times; twice using 1100-psi burst disks, then twice using 1300-psi burst disks.
5 Chamber vacuum 26 in Hg
6. Microcarriers. 1 0 μm, Au (*see* **Note 4**)

## 3.3. Bombardment of Embryos

1 Turn on the PDS1000/He, the vacuum pump, and the helium Adjust the pressure at the He bottle to 1300 psi
2 Assemble rupture-disk assembly with an 1100-psi rupture disk
3 Assemble microcarrier launch assembly: Place a sterile stop screen into position in the stopping screen support, place a loaded macrocarrier (particle side down) in the fixed nest, and screw the macrocarrier cover lid on until snug. Insert launch assembly into the second slot from the top
4. Center a plate of embryos on the target platform, slide it into the second slot from the bottom, remove the culture plate lid, and close the chamber door
5 Draw vacuum to approx 26 in Hg
6 Press the "fire" button until the rupture disk bursts
7. Release the vacuum, open the chamber door, put the lid back on the plate of embryos, and remove the plate and target platform from the chamber
8. Disassemble the microcarrier launch assembly, discarding the spent macrocarrier and stop screen
9. Disassemble the rupture disk assembly and discard the spent rupture disk
10 Repeat **steps 2–9** for each additional 1100 psi bombardment. Bombard each plate of embryos twice at 1100 psi, rotating the plates 90° between shots.

11 Adjust the pressure at the helium bottle to 1500 psi and repeat the above process using 1300 psi rupture disks, until all the embryos have also been bombarded twice at 1300 psi
12. Seal the plates with parafilm and incubate overnight at 26°C in the dark

### 3.4. Selection and Regeneration

1 The day after bombardment, the embryos should be transferred to LS2.5 medium supplemented with 4 mg/L bialaphos (*see* **Note 5**)
2 After incubation for 3 wk (26°C, dark), growth of callus should be apparent on the surface of many of the embryos The clumps of cells present in these calli should be subcultured to fresh selection plates During subculture, gently tease the clumps of cells apart, if possible Also, take care to assure good contact between the tissue and the medium, since crossprotection of bialaphos-sensitive cells is a potential problem (crossprotection can also be a problem when hygromycin is used as a selective agent, *see* **Note 6**). Also, because of the high frequency of siblings from individual embryos, it is advisable to subculture all calli from each embryo separately.
3 Subculture surviving tissue at least three consecutive times, with approx 3 wk intervals between subcultures The calli should be exposed to bialaphos for a minimum of 8 wk.
4 Subculture ~10–50 mg (~2–3 mm diameter) pieces of bialaphos-resistant callus onto MSD4 medium Incubate at 26°C in the dark (*see* **Note 7**)
5 10–14 d later, organized structures should be visible on the surface of the calli
6 After a total of 3–4 wk on regeneration medium, etiolated shoots and possibly roots will develop
7. Leave cultures in the dark until leaves develop (another 1–2 wk)
8 Transfer to a lighted incubator (26°C, 18 h light·6 h dark)
9 Leaves will green-up in a few days, but do not transfer developing plantlets to Magenta boxes until roots develop (*see* **Note 8**).
10 Collect leaf samples for PCR analysis during transfer of plantlets to Magenta boxes (*see* **Note 9**)
11. Transfer each individual plantlet to a Magenta box by picking it up with a pair of forceps and scraping any adhering callus off with another Insert the base of the plantlet into the medium far enough to submerge at least some of the roots.
12. Once transplanted into Magenta boxes, the plants grow quite rapidly After 2–3 wk, most plantlets will have developed a vigorous root system, grown leaves that touch the top of the box, and be ready to transplant to soil
13 Discard plantlet if PCR analysis indicates it lacks the gene of interest

### 3.5. Potting Plants and Growth Conditions

1 Prepare potting soil at least 12 h in advance to allow thorough wetting Mix five parts shredded, pasteurized peat moss with seven parts coarse vermiculite and saturate with Mikkelson's nutrient solution.

2. Insert a 7 × 7-in nylon mesh into the bottom of a 1-gal pot, and fill with potting soil to within 4 mm of the rim
3 Make a small depression in the center of the potting soil
4. Carefully remove a transgenic plant from a Magenta box
5 Remove all tissue-culture medium from the roots by rinsing them under running water
6. Remove any callus tissue or dead material from the roots and the base of the plant
7 Place the plant in the depression with the roots spread out, cover the roots with soil, and gently, but firmly, press down, so that roots and soil make good contact
8 Label the pot and loosely cover the plant and top portion of the pot with a 1-gal plastic bag
9. Immediately place the pot into a tray of water.
10 Place plant in a cool, low-light growth chamber (25°C day, 23°C night; 20,000 lux, 14 h light.10 h dark cycle). Replenish water in tray as needed to keep it full
11 After 2 wk, cut the corners off the plastic bag (leaving approx 2-in openings) to lower the humidity inside
12 3 wk after potting, remove the bag and begin fertilizer regimen
   a For the first 30 d, the plants should be top-fed with 0 1X Mikkelson's solution (*see* **Table 1**) Alternatively, Peters Professional Hydro-Sol fertilizer, as modified ***(18)***, can be used
   b Thereafter, bottom feed by topping the tray off, as needed, with full-strength (1X) Mikkelson's solution or fertilizer mix
13. After at least another week (4 wk total) under low-light conditions, adjust the growth chamber to high light (30,000 lux, 16 h light 8 h dark cycle) for at least a week, to complete acclimation During this period, the very fine leaves produced in the Magenta box die, and broader, normal leaves develop. Confirmation of bialaphos resistance can be tested after the plants have adapted to the soil (*see* **Note 10**)
14 Preferably, transfer plants to a greenhouse The plants should flower in ~8 wk after potting to soil, and the seeds will be mature ~30 d after pollination (*see* **Note 11**)

## 4. Notes

1. Since seed development is more advanced at the top of the panicles than the bottom, only ~30–40% of the seeds from each panicle will be at the correct stage Therefore, for each 100 embryos needed, collect three panicles
2 The final precipitation of the DNA before coating the particles must be done under aseptic conditions The DNA volume added to coat the particles may be critical ***(4)***

   The ratio of selectable marker DNA to gene of interest DNA and the total amount of DNA to coat onto the particles are parameters currently being optimized There are tradeoffs to be considered when modifying either parameter We expect that a higher proportion of gene of interest DNA will result in an

increase in the frequency of herbicide-resistant plants that also contain the gene of interest However, adding more of this DNA may also result in an increase in the number of copies of the gene of interest in each transformant Consideration of this eventuality is especially relevant, because of the increasing number of reports indicating that multiple copies of an introduced DNA sequence can lead to gene silencing by cosuppression, methylation, and DNA rearrangement and deletion (for reviews of these phenomena, *see* **refs. *19–21***)

3 When done correctly, the particles will be evenly distributed over the center of the macrocarrier. This step is tricky, and, to a large extent, will determine how evenly the particles will be distributed over the target area after bombardment. It is especially important to be fast, since the particles will settle very quickly, even in the pipet tip

4. We have had significantly more success using gold particles than the much less expensive tungsten particles However, acid pretreatment has been reported ***(22)*** to greatly increase the transformation frequencies obtained using tungsten particles We have confirmed this observation in transient expression experiments, and we are currently testing it in stable transformation experiments

5 Bialaphos is dissolved in water at a concentration of 4 mg active ingredient/mL and is stable for months at –20°C To the best of our knowledge, unformulated bialaphos is not currently available commercially. We obtain bialaphos through the courtesy of Dr Hiroyuki Anzai, Meiji Seika Kaisha, Morooka-cho, Kohoku-ku, Yokohama, 222 Japan

6 Chimeric plants can be obtained, presumably because of crossprotection ***(23)*** Therefore, it is imperative to apply stringent selection for a minimum of 2 mo

7 If problems are encountered in regenerating plants, subculture the resistant calli onto hormone-free LS2 5 without bialaphos for 7–10 d after the selection regimen, then transfer them to MSD4

8 Some plants without roots can be transferred, if the aerial portion of the plant is well-developed. They will usually develop roots quickly in the hormone-free medium.

9 To sample plantlets, remove the tissue-culture lid and position the upper 1 5–2 cm of a leaf inside a 1 5-mL microcentrifuge tube. Shear the leaf off by closing the lid while holding the lower portion of the leaf with forceps (to avoid pulling the plant up during sampling). As with all samples collected for PCR analysis, extreme care must be taken to avoid crosscontamination Store samples at –20°C until they are used for DNA isolation.

10 After the putative transgenic plant has been transplanted to soil and developed 4–5 leaves, a simple way to test for functional expression of the *bar* gene is to dip the end 3–4 cm of a leaf into an Herbiace solution (250 mg Herbiace/100 mL $H_2O$) and allow the leaf to dry. After 5 d under normal growth conditions, the reaction can be scored (cool, low-light conditions delay the response). If resistant, the plant will not suffer any damage If sensitive, only the leaf tissue directly exposed to Herbiace will brown and die, but the rest of the plant will remain unaffected. Always include a bialaphos-sensitive control for comparison

**Table 2**
**Pesticides Frequently Used for Rice**

| Trade name | Active ingredient | Manufacturer |
|---|---|---|
| Benomyl | Methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate | Miller Chem. and Fertilizer<br>Box 333<br>Hanover, PA 17331 |
| Banner | Propiconazole 1-([2-{2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl] methyl-1H-1,2,4-triazole) | Ciba-Geigy<br>P O Box 18300<br>Greensboro, NC 27419 |
| Avid | Avermectin $B_1$ (2 1%) | Merck MSD AGVET Division<br>126 East Lincoln Avenue<br>Rahway, NJ 07065 |
| Diazinon Knox Out 2FM | Diazinon. *O,O*-Diethyl *O*-(6-methyl-2-[1-methylethyl]-4-pyrimidinyl)phosphorothioate | Pennwalt, Agchem Division<br>Three Parkway<br>Philadelphia, PA 19102 |
| Pentac | Dienochlor (Decachloro *bis* [2,4-cyclopentadiene-1-yl]) | Sandoz Crop Protection<br>341 E Ohio Street<br>Chicago, IL 60611 |
| Stirrup | Insect pheromones | Fermone<br>2620 N 37th Drive<br>Phoenix, AZ 85009 |

11 Mite and insect infestations are problems common to greenhouse grown plants and heavy infestations can prevent seed set and production from otherwise healthy plants Grooming to remove all dead material and at least 6-in spacing between plants to allow air circulation will help reduce fungal and insect problems Benomyl or Banner (*see* **Table 2**), applied at the rates recommended by the manufacturers, can be used as needed to treat fungal infections To control mite or insect infestations, we have found Avid, Diazinon Knox Out 2FM, and Pentac applied at the manufacturers' recommended rates (**Table 2**) to be the most effective For prophylactic treatment, we cycle through the following at 2-wk intervals Avid, Diazinon, Diazinon, Diazinon, Pentac

In the event of severe mite infestations.

a Make the following solution. 25 mL Avid, 3 7 mL Stirrup-M (a behavior-modifying chemical) (**Table 2**), 2 7 g Benomyl, 30 mL of a mild dish washing liquid (e g , Ivory), and 8 L water
b Spray the plants to thoroughly wet them, especially around emerging leaves
c Place the plant and pot totally inside a large plastic bag and seal it
d Incubate the plants in a cool (21°C), shady place
e Remove the bags after 2 d if the plants are healthy and vigorous, or after 1 d if they are weaker and less healthy

f. Return plants to normal growth area
g Repeat the process 1 wk later, except substitute 5 mL Pentac for the Avid in the solution

The plants will show signs of stress (yellowing of outer leaves), but top fertilizing with an extra 250 mL of double-strength fertilizer/plant/week for a few weeks helps them recover Return to normal biweekly insecticide treatments

## Acknowledgments

Support from the Rockefeller Foundation Rice Biotechnology Program, the Texas A&M Office of Sponsored Projects, TAES Research Enhancement Program, and the Texas Advanced Technology Program (Project no 010366-108, no. 010366-149) is gratefully acknowledged. We thank many present and former members of the lab for their contributions to the techniques described here, especially Michael Battraw and Ginger Stuessy We also thank M. O. Way and Shannon Pinson, Texas Agriculture Experiment Station (TAES), Beaumont, for advice on rice pest control and plant growth conditions, respectively.

## References

1 Herdt, R W (1991) Research priorities for rice biotechnology, in *Rice Biotechnology* (Khush, G S and Toenniessen, G. H., eds.), Alden, Oxford, pp 19–54
2 Huntley, C C and Hall, T C (1996) Interference with brome mosaic virus replication in transgenic rice *Mol Plant–Microbe Interact* **9,** 164–170
3 Sanford, J. C., Klein, T M., Wolf, E D , and Allen, N (1987) Delivery of substances into cells and tissues using a particle bombardment process *Part Sci Technol* **5,** 27–37
4 Sanford, J C , Smith, F D , and Russell, J. A. (1992) Optimizing the biolistic process for different biological applications, in *Recombinant DNA, Part H*, vol 217 (Wu, R , ed ), Academic, San Diego, CA, pp 483–509
5 Heiser, W (1992) Optimization of Biolistic Transformation Using the Helium-Driven PDS-1000/He System Bio-Rad, Hercules, CA
6 Hoshikawa, K (1989) The growing rice plant an anatomical monograph Nobunkyo, Tokyo
7 Li, L , Qu, R , de Kochko, A , Fauquet, C , and Beachy, R N (1993) An improved rice transformation system using the biolistic method *Plant Cell Rep.* **12,** 250–255.
8 Shimamoto, K , Terada, R T , Izawa, T , and Fujimoto, H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts *Nature* **238,** 274–276
9. Toryama, K , Arimoto, Y , Uchimiya, H , and Hinata, K (1988) Transgenic rice plants after direct gene transfer into protoplasts *Biotechnology* **6,** 1072–1074
10 Hayashimoto, A , Zhijian, L , and Murai, N. (1990) A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants *Plant Physiol* **93,** 857–863

11 Battraw, M and Hall, T C (1992) Expression of a chimeric neomycin phosphotransferase II gene in first and second generation transgenic rice plants *Plant Sci* **86,** 191–202
12 Hiei, Y., Ohta, S., Komari, T , and Kumashiro, T (1994) Efficient transformation of rice (*Oryza sativa* L ) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA *Plant J* **6,** 271–282
13. Dong, J J , Teng, W M , Buchholz, W G , and Hall, T C (1996) *Agrobacterium*-mediated transformation of Javanica rice *Mol Breeding* **2,** 267–276
14. Linsmaier, E. and Skoog, F (1965) Organic growth factor requirements of tobacco tissue cultures *Physiol Plant* **18,** 100–127
15 Murashige, T and Skoog, F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures *Physiol Plant* **15,** 473–497
16 Dekeyser, R , Claes, B., Marichal, M., Van Montagu, M , and Caplan, A (1989) Evaluation of selectable markers for rice transformation *Plant Physiol* **90,** 217–223
17 Mikkelson, D (1987) University of California, Davis, personal communication
18 Battraw, M J. and Hall, T C (1990) Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants *Plant Mol Biol* **15,** 527–538
19 Flavell, R B (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication *Proc Natl Acad Sci USA* **91,** 3490–3496
20 Finnegan, J and McElroy (1994) Transgene inactivation: plants fight back! *Biotechnology* **12,** 883–888.
21 Matzke, M. A and Matzke, A J. M. (1995) How and why do plants inactivate homologous (trans)genes? *Plant Physiol* **107,** 679–685
22. Tomes, D. (1994) Pretreatment of microprojectiles prior to using in a particle gun Pioneer Hi-Bred International, International Patent no W094/17195
23 Kumpatla, S. P (1997) Transgene integrity, chimerism, silencing and stability in rice Ph D Thesis, Texas A&M University

# 41

# Molecular Analysis of Transgenic Rice

**Wallace G. Buchholz, James P. Connell, Siva P. Kumpatla, and Timothy C. Hall**

## 1. Introduction

It makes little sense to invest time and effort in assaying biological activities of transgenic plants if the plants do not contain a functional gene of interest. Although this appears to be self-evident, many investigators attempt to use biological assays for the presence of the gene of interest prior to conducting informative molecular characterization of transgenic plants. Where facile tests are available, such as those for β-glucuronidase (GUS) activity, this may be feasible. However, low levels of activity frequently make simple biochemical screening approaches unreliable Of the several analytical techniques available, we have chosen to describe methods for polymerase chain reaction (PCR) and genomic DNA blot analysis: methods that are fundamental for confirming and characterizing gene insertion

### *1.1. PCR*

PCR is a powerful and extremely sensitive technique for detecting DNA sequences in transgenic plants. PCR uses the activity of a (thermostable) polymerase, combined with oligonucleotide primers, substrates (DNA template, dNTPs, and so on), and cofactor, to preferentially synthesize and, hence, amplify a specific fragment of target DNA One cycle of a reaction generally consists of sequential incubation at high temperature (e.g., 94°C, 45 s) to denature the double stranded DNA substrate, incubation at a lower temperature (e.g., 50—65°C, 45–60 s) to allow the oligonucleotide primers to anneal, and incubation at 72°C (e.g , 2 min) to allow chain elongation. A major variable in the reaction is the annealing temperature; it must be low enough to allow the primers to anneal to the complementary sequences in the target DNA, but high

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

enough to prevent annealing at related sequences Repetition of this cycle 20–40 times results in amplification of target sequences between the primers, thereby providing a very sensitive method of detection However, the high sensitivity of this reaction makes false positives caused by contamination a serious problem (*see* **Note 1**).

### 1.2. Genomic Blot Analysis

Genomic DNA blot analysis, originally described by Southern *(1)*, can provide extensive information about the transformation event being studied. Important controls include the use of DNA from a nontransformed plant to show if sequences homologous to those of the probe being used are present in the plant genome, and large (>50 kb) DNA not treated with any endonuclease to confirm integration into chromosomal DNA. Detection of hybridizing bands of a size corresponding to the plasmid used for transformation provides cautionary information regarding the putative transformation The choice of restriction digests that will yield a predicted gene fragment are important for determining if rearrangement has occurred, and also provide insight to copy number. The use of an endonuclease that cuts only once within the gene of interest is valuable, since this permits determination of copy number and distinction of independent transformants Hybridization patterns are most simple to interpret if the probe used corresponds to a sequence that lies entirely to one side of the single restriction site

The DNA isolation protocol presented is a slightly modified version of the method developed by Taylor et al. *(2)*. Plant tissue is frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder is added to an extraction buffer composed of CTAB detergent and a high concentration of salt, in which the nucleic acids (NAs) are solubilized and subsequently separated from cell walls and other particulates by centrifugation Extraction of the solution with an organic solvent removes many carbohydrates and proteins. Upon addition of the precipitation buffer that dilutes the salt concentration, an insoluble NA.CTAB complex forms. After centrifugation, the NA:CTAB precipitate is solubilized under high-salt conditions and the NAs are selectively precipitated with isopropanol. RNA is removed by RNase digestion, and, after additional alcohol precipitations, DNA of sufficient purity to be digested with restriction enzymes has been isolated.

Hundreds of variations of genomic blot analysis are currently used with success. The method described below relies on a minimum amount of equipment that might be available in all laboratories Genomic DNA is size fractionated by agarose gel electrophoresis. The DNA is made single-stranded by base denaturation and is transferred to a membrane by capillary action. Heat treatment under vacuum is then used to bind the DNA to a mem-

**Table 1**
**10X PCR Buffer (for 10 mL)**

| For a final (10X) concentration of | use | of a. |
|---|---|---|
| 100 m*M* Tris-HCl (pH 8 0) | 1 mL | 1*M* Tris (pH 8 0) |
| 500 m*M* KCl | 2 mL | 2.5*M* KCl |
| 15 m*M* $MgCl_2$ | 0 75 mL | 0 2*M* $MgCl_2$ |
| 0.1% Gelatin | 10 mg | Powdered gelatin |
| 2 m*M* dATP | 0 2 mL | 0.1*M* dATP |
| 2 m*M* dGTP | 0 2 mL | 0 1*M* dGTP |
| 2 m*M* dTTP | 0 2 mL | 0 1*M* dTTP |
| 2 m*M* dCTP | 0 2 mL | 0 1*M* dCTP |
| 1% Triton X-100 | 0.1 mL | 100% Triton |
| $H_2O$ | 5 35 mL | 55 55*M* Stock |
| **Total** | **10.0 mL** | |

brane. Radiolabeled single-stranded DNA probe is hybridized to complementary sequences present in the filter bound DNA Nonspecifically bound probe is washed off under conditions that allow specifically bound probe to remain hybridized. The presence of complementary sequences is then visualized by autoradiography.

## 2. Materials for Molecular Analyses

### 2.1. Isolation of DNA for PCR Analysis

1 Disposable pellet pestles (e g , no 749520, Kontes Glass, Vineland, NJ)
2. Extraction buffer: 200 m*M* Tris-HCl, pH 8 0, 250 m*M* NaCl, 25 m*M* Ethylenedinitrilotetraacetic acid (EDTA), 0 5% sodium dodecyl sulfate (SDS)
3 Isopropanol (IpOH)
4 TE 10 m*M* Tris-HCl, pH 8 0, 1 m*M* EDTA

These additional reagents are needed if DNA is being isolated from callus tissue·

5 5*M* NaCl.
6 5*M* KOAc.
7 30% polyethylene glycol 8000 (no P 5413, Sigma, St Louis, MO) in TE.

### 2.2. Supplies for PCR Reactions

1 PCR buffer (*see* **Table 1**)
2 Sense and antisense primers
3 Thermostable polymerase (e g , AmpliTaq, Perkin-Elmer, Norwalk, CT)
4. Mineral oil
5 Thermocycler (e g , PTC-100, MJ Research, Watertown, MA)

## 2.3. Isolation of DNA for Genomic Blot Analysis

1 Extraction buffer (Stable for months at room temperature) 2% Hexadecyltrimethylammonium bromide (CTAB, no. 5882, Sigma), 100 m*M* Tris-HCl, pH 8 0, 20 m*M* EDTA, 1.4*M* NaCl, 2% β-mercaptoethanol added just prior to use
2 Precipitation buffer (stable for months at room temperature) 1% CTAB, 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 1% β-mercaptoethanol added just prior to use
3 Mortar and pestle
4 Liquid nitrogen ($N_2$)
5 Shaking water bath or hybridization oven (optional)
6 Chloroform.isoamyl alcohol 24 1
7 Polypropylene oak ridge (or similar) centrifuge tubes
8. Polypropylene tubes (17 × 100 mm) with caps
9 1*M* Ammonium acetate
10 7 5*M* Ammonium acetate
11 Isopropanol
12 TE + RNase 10 m*M* Tris-HCl, pH 8 0, 1 m*M* EDTA, 20 μg/mL RNase A
13 Ethanol (EtOH, 70%)

## 2.4. Supplies for Genomic Blot Analysis

### 2.4 1. Agarose Gel Electrophoresis

1 40X Tris-acetate buffer (stable for months at room temperature) 193 5 g Trizma, 65 7 g sodium acetate (anhydrous), 29 8 g EDTA, 650 mL $ddH_2O$, adjust pH to 8.0 with glacial acetic acid, adjust volume to 1 L
2 Loading buffer (100 mL) (filter-sterilize, stable for months at room temperature) 0 1 g bromophenol blue, 15 g Ficoll 400, to 100 mL with $ddH_2O$
3 Peristaltic pump to recirculate buffer in gel box
4 Orbital shaker (e.g., Model G2, New Brunswick Scientific, Edison, NJ)
5 Ethidium bromide solution (0 5 μg/mL in $ddH_20$)
6 Pyrex dish.
7 UV light box (preferably 302 nm)
8. UV germicidal lamp (254 nm) or UV crosslinker (e.g , Stratalinker, Stratagene, San Diego, CA)
9 Camera equipped with filters (i e , UV haze and Wratten 23a filters) for documentation

### 2.4.2 Capillary Blotting

1. Nitrocellulose (e.g., no BA 85, Schleicher and Schuell, Keene, NH) or nylon membrane (e g., Hybond N, Amersham, Arlington Heights, IL)
2. Chromatography paper (e.g., no. 3030917, Whatman, Maidstone, UK)
3 Blotting table (*see* **Note 2**)
4. Paper towels cut to the size of the gel to be blotted
5 Heat sealable plastic bags (e g., Seal-A-Meal bags, Dazey, Industrial Airport, KS)
6 50X Denhardt's solution (100 mL, can store for months at –20°C) 1 g BSA (fraction V), 1 g Ficoll 400, 1 g polyvinylpyrrolidone (mol wt ~40,000), to 100 mL with $ddH_2O$, mix extensively to dissolve

7. 20X SSC (1 L, can store for months at room temperature). 175 g NaCl, 88.23 g Na citrate, to 1 L with $ddH_2O$.
8. Southern base (make fresh before use): 850 mL $ddH_2O$, 20 g NaOH, 87.7 g NaCl (after NaOH has dissolved), adjust volume to 1 L
9. Southern neutralization solution (1 L, can store for months at room temperature) 800 mL $ddH_2O$, 60.5 g Trizma base, 175.3 g NaCl, adjust pH to 7.0 with HCl, adjust volume to 1 L
10. Prehybridization solution (100 mL, can store for months at –20°C) 30 mL 20X SSC, 20 mL 50X Denhardt's solution, 50 mL $ddH_2O$
11. Hybridization solution (10 mL, can store for months at –20°C): 3 mL 20X SSC, 1 mL 50X Denhardt's, 200 µL 1*M* Tris-HCl, pH 8.0, 100 µL 10% SDS, 100 µL 0.5*M* EDTA, 250 µL 0.1*M* Na pyrophosphate, 5.35 mL $ddH_2O$
12. Salmon sperm DNA (10 mg/mL in water) (*see* **Note 3**)
13. 6X Wash mix (1 L, can store for months at room temperature) 300 mL 20X SSC, 10 mL 20% SDS, 10 mL 0.5*M* EDTA, 680 mL $ddH_2O$
14. 0.3X Wash mix (1 L, can store for months at room temperature) 15 mL 20X SSC, 5 mL 20% SDS, 980 mL $ddH_2O$.
15. Scientific imaging film (e.g., Kodak X-OMAT XAR-5, Eastman Kodak, Rochester, NY).

## 3. Methods

### 3.1. Extraction of DNA for PCR Reactions

#### 3.1.1. DNA Extraction from Leaves for PCR

Unless otherwise noted, all steps should be carried out at room temperature

1. Add liquid nitrogen to a collected leaf sample (e.g., *see* Chapter 40, **Subheading 3.4.**) and grind to a fine powder in a 1.5-mL microcentrifuge tube with a pellet pestle
2. Add 300 µL of extraction buffer, mix, and incubate extract in a water bath (65°C, 30–60 min)
3. Centrifuge briefly (15,000*g*, 30 s) to pellet cellular debris
4. Transfer 150 µL of supernatant to another 1.5-mL microcentrifuge tube
5. Add 150 µL of isopropanol and mix by inversion
6. Incubate at room temperature for at least 5 min
7. Centrifuge (15,000*g*, 5 min) to pellet precipitate
8. Aspirate and discard each supernatant with a fresh pipet tip
9. Dry pellets briefly under vacuum
10. Dissolve each pellet in 30–50 µL TE (depending on the size of the DNA pellet)

#### 3.1.2. DNA Extraction from Callus for PCR

This is a modification of the method of Agudo et al. *(3)*. If scaled up, sufficient quantities of digestable DNA can be isolated from callus for genomic blot analysis.

1. Put 10–100 mg of callus tissue in a 1.5-mL microcentrifuge tube, add 300 µL of extraction buffer, and homogenize, using a pellet pestle
2. Incubate the extract in a water bath (65°C, 30–60 min)

3 Centrifuge briefly (15,000*g*, 30 s) to pellet cellular debris
4. Transfer 300 µL of supernatant to another 1.5-mL microcentrifuge tube
5 Add 75 µL 5*M* NaCl and 120 µL 5*M* KOAc and incubate on ice 5–10 min
6. Centrifuge (15,000*g*, 5 min) to pellet precipitate
7. Transfer supernatant to another 1 5-mL microcentrifuge tube
8 Add 180 µL 30% PEG to the supernantant fraction, mix and incubate on ice for at least 20 min
9 Centrifuge (15,000*g*, 5 min) to pellet nucleic acids
10 Aspirate and discard supernatant with a fresh pipet tip
11 Dry pellets briefly under vacuum
12 Dissolve each pellet in 30–50 µL TE (depending on the size of the DNA pellet)

### 3.2. Running the PCR Reactions

Prior to analyzing putative transgenic rice plants, primers should be designed and synthesized (*see* **Note 4**). Also, a PCR amplification program should be optimized (*see* **Note 5**). The following steps should be done sequentially in order to limit possibilities of contamination.

1 Set up a single homogenous reaction mix that contains the following components (per sample) Always include negative and positive control reactions (*see* **Note 6**) Reaction mix required per sample. 5 µL 10X PCR buffer, 2 µL sense primer @ $OD_{260}$ = 2, 2 µL antisense primer @ $OD_{260}$ = 2, 0 05 µL *Taq* DNA polymerase (0 25 U), 40 µL $H_2O$, yielding a total volume of 49 µL per sample
2 Aliquot 49 µL of mix into a 0.5-mL microcentrifuge tube for each sample, and overlay with 25 µL of mineral oil (Put all PCR stock solutions back into the –20°C freezer before handling any DNA samples )
3 Add 1 µL of rice DNA for each reaction. (Put away all DNAs from test samples before handling any positive control DNA )
4 For the positive-control reaction, add 10 pg (1 µL of a 10 ng/mL) plasmid DNA to ~100 ng of wild-type rice DNA
5 Run the appropriate PCR program for fragment amplification
6 Transfer 15 µL of the PCR reaction to another tube containing 3 µL of gel loading dye
7 Size-fractionate PCR products by agarose gel electrophoresis (1–2% gel, depending on the size of the amplification product expected)
8 Results of typical reactions are shown in **Fig. 1** and discussed in **Section 3.5.1.**

### 3.3. Extraction of Genomic DNA for Blotting

The volumes in this protocol are adjusted for 0.5–1.5 g samples and will yield 50–100 µg DNA. For larger samples, use appropriately scaled-up volumes Unless otherwise noted, all steps should be carried out at room temperature.

1 Weigh out approx 1 g of rice leaves (rapidly growing tissues such as young leaves are preferable) and slice into 0 5-cm sections Place leaf cuttings into a cold mortar, add enough liquid nitrogen to cover the leaves and grind them to a

fine powder with a pestle Add additional liquid nitrogen, as needed, to keep the tissue frozen.

2 Add frozen powder to 5 mL extraction buffer (preheated to 65°C) in a polypropylene screw-cap tube (e.g., Oak Ridge tube) and mix well

3 Incubate at 65°C for at least 30 min (2–3 h is optimal) Rolling (e.g , in a hybridization oven) or gently mixing during incubation significantly improves DNA yield

4. Cool samples to <50°C and add an equal volume of chloroform·isoamyl alcohol (24 1) and mix gently by inversion for ~2 min Be sure to relieve pressure in the tubes by loosening the caps occasionally

5 Centrifuge (10 min, ~2000*g*)

6. Transfer the supernatant (~3 mL) to a 17 × 100-mm (e g , Falcon 2059) tube and add 2 vol of precipitation buffer.

7 Invert to mix and incubate (room temperature, 30 min). If solutions do not become cloudy, add another 1 mL of precipitation buffer and incubate longer For dilute solutions, it is advisable to incubate overnight

8 Centrifuge (5 min, 2000*g*) to pellet nucleic acids

9. Discard supernatant. (**Caution:** The pellets are quite friable at this stage and are easily lost!) Aspirate as much of the remaining liquid as possible

10 Dissolve the pellet in 200 µL of 1*M* $NH_4OAc$. Sometimes the pellets resist dissolving and hence this step can be more of a resuspension In either case, transfer the nucleic acids to 1 5-mL microcentrifuge tubes

11 Add 100 µL $NH_4OAc$ (7 5*M*) and mix well

12 Add 1 0 mL of isopropanol and mix well by inversion

13 Incubate at room temperature for 5 min

14 Centrifuge (15,000*g*, 5 min) to pellet precipitate.

15 Aspirate the supernatant and dissolve the pellet in 200 µL TE + RNase. At this point the pellet should dissolve easily. If not, or if the solution is extremely viscous, add more TE + RNase

16 Incubate for 1–2 h at 37°C

17 Add 100 µL $NH_4OAc$ (7 5*M*) and mix.

18. Add 500 µL isopropanol and mix well by inversion. The nucleic acids may be visible as a fluffy, brownish-white precipitate.

19 Centrifuge (15,000*g*, 5 min) to pellet precipitate

20 Rinse pellets with 70% EtOH and briefly dry

21 Dissolve pellets in 100µL of TE

22. Quantify DNA by fluorometry Spectrophotometric analysis can be accurate, but may not be so because of contaminants. Typically, the DNA concentration will be ~0 5–1 0 mg/mL.

## *3.4. Genomic DNA Blot Analysis*

### *3.4.1. Preparation of DNA for Blotting*

1 Digest DNAs to completion with the appropriate restriction enzyme(s). At least the first few times DNA is isolated with this method, it is useful to confirm it has

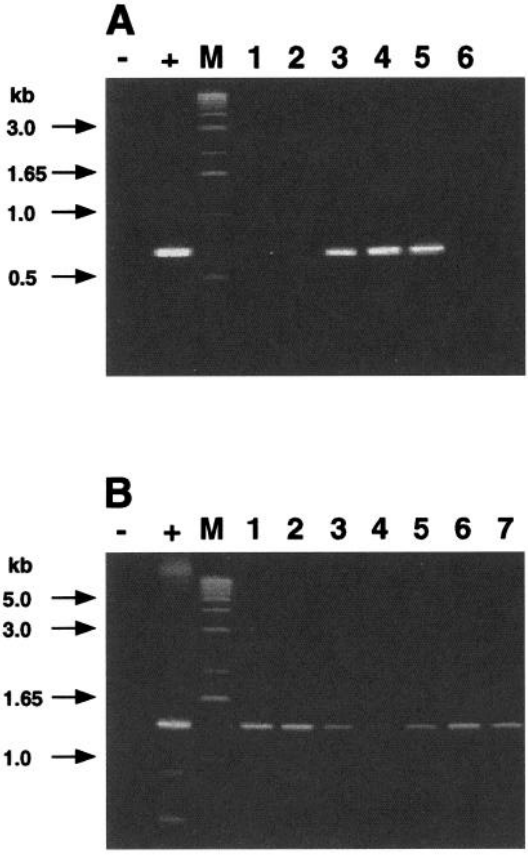



Fig. 1. PCR analysis of putatively transformed rice. DNA for PCR amplification was extracted from rice leaves and amplified as described in the text. The PCR products were size fractionated by electrophoresis through 1.2% agarose gels cast in Tris-borate buffer. **(A)** PCR amplification products obtained using green fluorescent protein gene (*gfp*)-specific sense (GFPS117; 5'-GAACTTTTCACTGGAGTTGTCC-3') and antisense (GFPA817: 5'-TATTTGTATAGTTCATCCATGC-3') primers. The PCR program (94°C, 30 s; 54°C, 30 s; 72°C, 60 s) was run for a total of 30 cycles. DNAs used as substrate in each lane were: –, reaction mix without added DNA; +, 10 pg *gfp* plasmid DNA mixed with 100 ng wild-type T309 DNA; 1–5, bialaphos-resistant transgenic rice cotransformed with chimeric *bar* ***(9)*** and *gfp* ***(10)*** genes; 6 and 7 T309 plants transgenic for a different gene of interest. Lane M contains BRL 1-kb ladder size standards. **(B)** PCR amplification products obtained using rice triose phosphate isomerase ***(11)*** gene-specific sense (TPIS4775: 5'-GAGACTCTCGAGCAGCGGG-3') and antisense (TPIA6122: 5'GCATTGCTCAAGCTGCAGG-3') primers. The PCR program (94°C, 30 s; 50°C, 30 s; 72°C, 60 s) was run for a total of 30 cycles. Lanes: As in A.

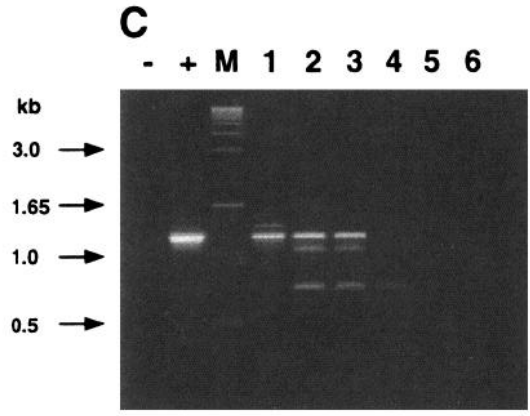



Fig. 1. **(C)** PCR amplification products obtained using β-glucuronidase gene-specific sense (GUSS400: 5'-GGTGGGAAAGCGCGTTACAAG-3') and antisense (GUSA1599: 5'-GTTTACGCGTTGCTTCCGCCA-3') primers. The PCR program used was the same as for A. DNAs used as substrate in each lane were: –, reaction mix without added DNA; +, 10 pg β-glucuronidase-containing plasmid DNA in 100 ng wild-type T309 DNA; 1–4, bialaphos-resistant transgenic rice cotransformed with *bar* and β-glucuronidase chimeric genes; 5 and 6, wild-type T309 plants. Lane*M* contains BRL 1 kb ladder-size standards.

been cut to completion by running a small aliquot on a gel before running the gel to be blotted (*see* **Note 7**).

2. Cast a 0.7% agarose gel in 1X Tris-acetate buffer. A higher concentration gel can be used if resolution of smaller fragments is desired.
3. Add 5–6 μL loading buffer to each DNA sample, load the gel and electrophorese. Recirculate the buffer, since 1X TAE has little buffering capacity. (In 1X TAE buffer, the dye front will migrate about 10 cm if run at 0.8 V/cm for ~16 h, i.e., 23 V in a BRL H5 gel box [for running 11 × 14 cm gels].)
4. Carefully transfer gel to a glass baking dish. Cut ~2–3 mm off each side and the bottom to remove the meniscuses. Remove the upper portion of the gel by cutting through the wells. Horizontally shave the meniscus off the upper surface of the gel at each well. Cut the top right corner off the gel to make orientation easy.
5. Add ethidium bromide solution (200–300 mL) and stain DNA by gentle agitation on an orbital shaker for 20–30 min.
6. Place the stained gel on a UV light box (302 nm), align a ruler with the wells beside a lane with size standards and photograph.
7. Introduce single-strand breaks into the high-mol-wt DNA with UV light (254 nm) (*see* **Note 8**).

8 Place gel back in the baking dish and add Southern base solution (use 1 L of solution for an 11 × 14-cm gel) and incubate with gentle agitation on an orbital shaker for 1 h (4-mm-thick gels) to 1 5 h (>4-mm-thick gels) to denature the DNA
9 Briefly rinse excess base from the gel a few times with $ddH_2O$
10 Add Southern neutralization solution (~500 mL) Gently agitate for 45 min, pour off, and replace with fresh neutralization solution Gently agitate for another 1 h (1 5 h for thicker gels).

### 3.4.2. Blotting Gel

1. Cut two pieces of chromatography paper and one piece of nitrocellulose or nylon membrane to the exact size of the gel to be blotted. Cut off the top right corner of the membrane and label the blot with a soft lead pencil on the bottom center (*see* **Note 9**)
2 Wet membrane in hot (60–90°C) $ddH_2O$. When hydrated, decant water and saturate membrane and chromatography paper with 20X SSC.
3 Assemble a blotting table (*see* **Note 2**).
4 Put a puddle (~20–30 mL) of 20X SSC on the blotting table and lay the gel upside down in the puddle, being careful to avoid trapping bubbles. Gently rub the gel, or roll with a pipet, to remove any bubbles trapped between the gel and the chromatography paper (*see* **Note 10**)
5 Put a puddle of 20X SSC on the gel Carefully lay the membrane (labeled side down) on the gel and align the cut corner for orientation and the top of the membrane precisely with the wells to allow accurate measurements Avoid trapping bubbles, as above.
6 Put a puddle of 20X SSC on the membrane. Carefully lay one piece of chromatography paper on the membrane, avoiding bubbles Lay the second piece on the first and then stack paper towels (that are cut to the same size as the gel) on top until the towels are 15–20 cm high (*see* **Note 11**).
7. Fill dish with 20X SSC, until the level is just to the bottom of the glass plate
8 Cover the dish and paper towel stack with plastic wrap to prevent evaporation If desired, a weight can be placed on the paper towels A 15 × 20-cm glass plate with a small water bottle balanced in the center is adequate (total weight ~400 g)
9 Blot for 24–48 h Replace wet paper towels (do not disturb the bottom 1–2 cm of the stack) and replenish the 20X SSC in the dish as needed
10. When blotting is complete, remove the paper towels. Leave the two sheets of chromatography paper with the nitrocellulose Lift the gel off the blotting table and turn it over. Mark or cut the membrane as needed, and remove and discard the gel. Note that the gel should be quite flattened. Evaluate transfer efficiency by staining the gel with EtBr, if desired
11. Dry the filter between sheets of chromatography paper and further dry for at least 2 h at 80°C, under vacuum. Be careful: Nitrocellulose will be very brittle at this stage. (Alternatively, while the membrane is still damp, but not shiny wet, and on the chromatography paper, place it with the DNA side up inside UV crosslinker and fix the DNA to the membrane )

12. Place the membrane in a sealable plastic bag (moisten nitrocellulose with 2X SSC first). Seal the bag very close to the membrane on three sides, but leave 3–4 cm of bag on the open end This extra space will allow the bag to be opened and resealed several times as solutions are removed or added
13 Add a generous volume of prehybridization solution (10–20 mL, depending on the blot size) and seal the bag close to the cut edge, excluding as many bubbles as possible
14 Prehybridize at 65°C for at least 4 h (overnight, if background has been a problem)
15. Cut the end off the plastic bag and squeeze the prehybridization solution out Rolling a pipet over the bag will get most of it out Seal the bag close to the edge and then cut a corner off to add the hybridization mix through
16 Prepare the hybridization solution:
    a Radiolabel the hybridization probe using any of the commercially available kits (e g , nick translation, random prime, and so on).
    b Use a minimum volume of hybridization solution (5–6 mL is usually adequate for an 11 × 14 cm membrane, use less if possible)
    c Just prior to use, add 2 µg denatured, sheared salmon sperm DNA/mL
    d Denature the hybridization probe (*see* **Note 12**) and add it to the hybridization solution
17. Add the hybridization solution to the bag and seal the bag as close to the membrane as possible, being careful to eliminate all the bubbles Two blots, placed back to back, can be hybridized in the same bag
18 Hybridize overnight at 65°C, preferably in a shaking water bath or on a rocker, to promote movement of the solution in the bag
19 Transfer the membrane from the bag and wash sequentially with ~200 mL each of
    a 6X wash mix, 65°C, 30 min;
    b 0 3X wash mix, 65°C, 30 min,
    c. 0 3X wash mix, 65°C, 30 min, and
    d. 2X SSC briefly at room temp to rinse SDS away
20 Place the blot between two layers of plastic wrap, and, if the blot will be reprobed later, care should be taken to keep the membrane moist (*see* **Note 13**)
21 Expose to imaging film

## 3.5. Interpretation of Results

### 3.5.1. Interpretation of PCR Results

**Figure 1** shows a variety of PCR results that were obtained using the above techniques. Details of the reactions are described in the figure legend. Panels A and B show very clean, predictable, and easy to interpret results; those in Panel C are less clear-cut, but quite common.

In panel A, the negative control lanes (–, 6 and 7) lack amplification products when the green fluorescent protein (GFP) primers were used, and the posi-

tive-control lane (+) shows a product of the predicted size. Taken together, these data indicate the results of this experiment should be valid. It can be concluded that the plants assayed in lanes 3–5, which show fragments of the predicted size, contain at least one unrearranged copy of the GFP coding region (between bp 117 and 817) However, it is not clear whether the results from plants assayed in lanes 1 and 2 are valid. If these two DNA extracts could serve as substrates for PCR amplification, the negative result would be valid. In contrast, if they could not support amplification, they could be false negatives. Therefore, all samples were tested for the ability to act as substrate for PCR by amplifying an endogenous single-copy gene (triose phosphate isomerase), as shown in Panel B.

In Panel B, the negative (–) and positive (+) controls gave the expected results, indicating the experiment should be valid. However, the positive control (TPI plasmid) resulted in the amplification of additional, unexpected fragments The very high-mol-wt fragments indicate the presence of rice DNA added to that reaction, and the smaller than predicted fragments are probably caused by nonspecific priming at other sites in the plasmid. This is likely because of the low annealing temperature used The results in lanes 1–7 indicate that all the samples, importantly, samples 1 and 2, can serve as substrate for PCR. Taken together with those in Panel A, these data provide strong evidence that samples 1 and 2 lack an intact copy of the central portion of the GFP coding region Be aware that this result does not necessarily indicate that genomic blot analysis of these plants (using GFP as a probe) would result in no hybridization, since rearranged or deleted portions of GFP could be present. Rather, it simply indicates that the two primer sites are not present or, if present, are not in the correct relative orientation

The presence and absence of fragments in the positive (+) and negative (–, 5 and 6) control lanes, respectively, validate the results of the PCR shown in Panel C Another reaction (data not shown) indicated all samples served as substrate for PCR Therefore, it can be concluded that the plants assayed in lanes 1–3 contain at least one unrearranged copy of the GUS coding region (between bp 400 and 1599) An additional higher mol-wt fragment (lane 1) and lower mol-wt fragments (lanes 2 and 3) are also present, probably indicating the presence of additional rearranged copies of the GUS coding region in these plants Furthermore, since the banding pattern is identical in lanes 2 and 3, it is likely that these two plants are siblings. The presence of a low-mol-wt fragment, but not a fragment of the expected size in lane 4, suggests this plant contains only a rearranged copy(ies) of the GUS coding region

### *3.5.2. Interpretation of Genomic Blot Results*

**Figure 2** shows a variety of genomic blot results that were obtained using the above techniques. The plants analyzed had previously been shown to be positive by PCR analysis. A complete set of analysis is shown for JKA plants 56 and 61 that had been cotransformed with construct JKA (**Fig. 2A**) and UBar (a chimeric bialaphos resistance gene driven by the maize ubiquitin 1 promoter, which was a gift of A. Christensen and P. Quail). The DNA fragment used as a hybridization probe is shown in panel A. First, the controls give the expected results; no hybridization is detected to DNA isolated from the nontransgenic, wild-type T309 (cv. Taipei 309); whereas it is detected to the four-copy positive-control reconstruction lane (4X) containing wild-type T309 DNA spiked with plasmid JKA DNA. Second, it is evident that sequences related to the probe are integrated in the genome of the transgenic plants, since all the hybridization seen comigrates with the high-mol-wt rice genomic DNA in those lanes containing undigested DNA (u).

When double-digested with *Bam*HI and *Eco*RI, plants 56 and 61 both contain the 3 2 kb fragment expected, if an unrearranged copy(ies) of the gene is present. This conclusion is confirmed by the presence of the expected 1.6 kb *Eco*RV fragments in both plants There appear to be many more intact copies of the *Eco*RV fragment than the *Bam*HI/*Eco*RI fragment, suggesting that many rearrangement events occurred in the promoter region between the *Eco*RV and *Bam*HI sites In addition to the expected fragments, many fragments of higher and lower mol wt are also present, indicating substantial rearrangement of the input plasmid. A rough estimate of the total number of copies of these sequences present in the rice genome can be made by estimating the number of fragments present (and their intensities compared to the reconstructions) in the lanes digested with *Kpn*I. A conservative estimate is that plant 56 has ~40–50 copies and plant 61 has well over 100 copies

In contrast to the high numbers of copies of the gene of interest in the two JKA plants shown, the WBD plants have relatively low numbers of copies These DNAs, including the one copy reconstruction, have been digested with *Eco*RI, which should release a 2.1-kb fragment if the 3' end of the promoter, the coding region for arcelin, and the complete nopaline synthase poly(A) addition site fragment are intact. A fragment of the correct size is present in plant 87, but not in the other two. It is unclear whether plants 68 and 76 have a functional copy(ies) of the introduced gene, with the limited data available from this single blot. For instance, if the 3' *Eco*RI site was lost during DNA rearrangement and integration, but the poly(A) addition signal was left intact, either plant could theoretically contain a functional gene. In contrast, if the *Eco*RI site in the promoter has been deleted because of rearrangement, it would

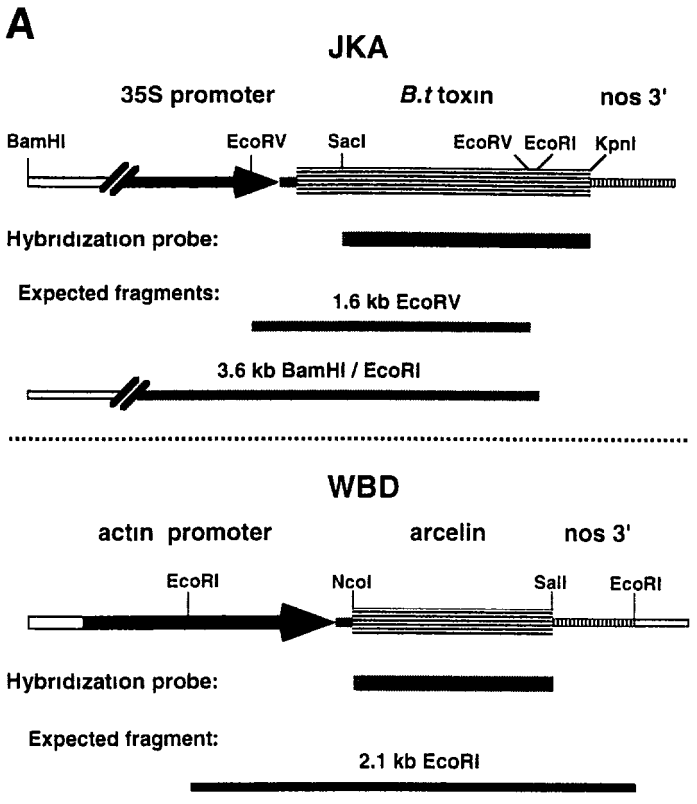



Fig 2 Genomic blot analysis of rice **(A)** Partial restriction maps of chimeric genes JKA and WBD that were introduced into rice The DNA fragments used as hybridization probes and the fragments expected are shown.

be likely the promoter was disrupted and the likelihood of a functional gene being present would be low. Although additional genomic blots using different restriction digests could be used to determine the extent and location of the rearrangements, it is easier to amplify overlapping fragments using PCR (e.g., *see* Fig. 7 in **ref.** *4*) to confirm the integrity of the gene.

In just the above few examples, it is easy to see that the copy number of introduced sequences can vary from low (1–2) to very high (>100). Because of the increasing number of reports of gene silencing *(5-7)*, which are often attributed to the plant responding to multiple copies of genes, a challenge for future research is to determine how to regulate and limit the number of copies introduced. One mechanism that might explain, at least partially, how the copy number varies was presented at a recent meeting *(8)* Scanning E*M* photos of DNA-coated microcarriers showed some that had small precipitates of DNA

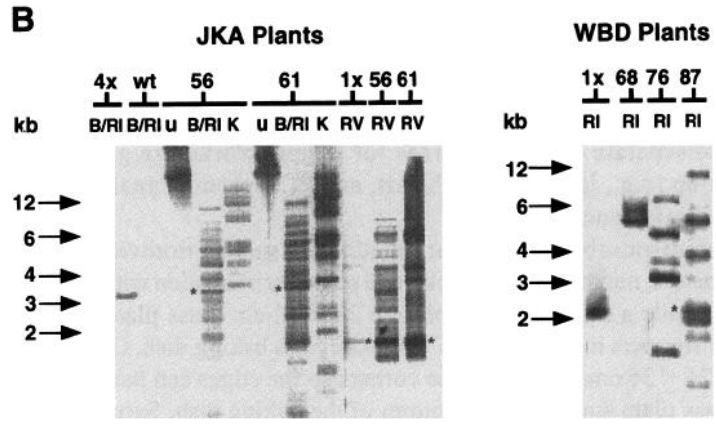



Fig. 2. **(B)** DNA from PCR positive transgenic plants was isolated and subjected to genomic blot analysis using the accompanying protocol, except only 1 μg of DNA was used for the JKA plants. The fragments indicated in panel A were random primer labeled with [$^{32}$P]dCTP and used as hybridization probes. Size markers are shown to the left of the blots and the expected fragments are indicated with asterisks. 4x or 1x, 4 or 1 copy reconstructions of the chimeric genes; wt, wild-type T309 DNA; 56 and 61, independently transformed rice plants transgenic for construct JKA; 68, 76, and 87, independently transformed rice plants transgenic for construct WBD. The indicated DNAs were left undigested (u) or digested with *Bam*HI and *Eco*RI (B/RI), *Kpn*I (K), *Eco*RV (RV), or *Eco*RI (RI).

attached; others had DNA precipitates attached that were actually larger than the 1-μm microcarriers.

## 4. Notes

1. Contamination of samples with plasmid DNA and crosscontamination during plant DNA isolation must be rigorously avoided. Since the reaction is exponential and the total amount of product made is primer-limited, the signal strength cannot be used to judge whether a fragment is caused by contamination. In theory, if the reaction is carried to completion, all reactions with the same primers should result in the same amount of product regardless of input DNA concentration. In practice, differences in signal intensity do occur, but are presumably the result of inefficient priming, inefficient elongation, inhibitors in the crude DNA samples, or a combination of these factors. Therefore, the following precautions should always be taken with all materials that will be used for PCR analysis.
   a. Use gloves at all times and frequently wash or change them.
   b. Use aerosol resistant pipet tips for all steps, including stock solution preparation, DNA isolations, and PCR reactions.

c Use PCR-designated reagents, preferably stored in small aliquots For those not aliquotted in advance (e g., organic solvents), aliquot into a new disposable chemical resistant container (e.g , Falcon 2059 tubes) just prior to use and discard any excess

d. Use separate designated areas for sample workup (e.g , lab bench), PCR set up (e g , laminar flow hood), and PCR product analysis (e g , electrophoresis bench)

e. Use disposable pestles for grinding tissue Periodically decontaminate pipeters, microcentrifuge racks, and so on, by irradiation with a germicidal lamp

2 To assemble a blotting table, place a 20 × 30-cm glass plate on four size 11½ rubber stoppers in a 26 5 × 37 5 × 5 5-cm glass baking dish Cut chromatography paper 26 × 36 cm, and notch the corners so the edges can hang over the sides of the glass plate and touch the bottom of the baking dish Saturate the paper with 20X SSC Cut another paper 20 × 30 cm and place over first on top of the glass Saturate with 20X SSC and roll any bubbles out with a pipet The table can be stored for weeks by sealing the top with plastic film. Replace the upper layer of paper prior to reuse

3 The salmon sperm DNA solution should be denatured and sheared by heating to 100°C for 10 min with intermittent vigorous vortexing It can be stored for months at –20°C. Once denatured, avoid conditions that allow renaturation and occasionally repeat heat denaturation

4 Most primers we use have 20–24 nt and are ~50–60% G + C Two of the last 3 nt at the 3' end of the primers, especially the ultimate nt, should be G or C if possible We generally amplify fragments that range from ~400–2000 bp

5 Optimization of a PCR program can be done by using 10 pg (1µL of a 10 ng/mL solution) purified plasmid DNA as substrate A good starting point for determining the annealing temperature ($T_a$) of a typical (i e , ~20 nt) PCR primer in standard PCR buffer is to use the equation $T_a$ = (4 × G + C residues) + (2 × A + T residues). Twenty cycles are usually adequate to amplify a fragment from purified plasmids. Once conditions are defined for amplification of the target sequences from plasmid DNA, add 10 pg of plasmid to 50–100 ng of wild-type rice DNA (isolated using the above protocol) and run the reaction for 30–40 cycles (This number of amplification cycles is usually required because of the relatively low purity of DNA prepared using the above method ) Further adjustments to the PCR program may be necessary.

6. For every set of PCR reactions, include two negative controls (no DNA and wild-type rice DNA) and a positive control. Preferably, the positive control DNA should be from a transgenic plant known to contain the target sequences Alternatively, plasmid added to wild-type rice DNA (~10 pg into 100 ng) can be used The DNAs used for controls should be isolated in parallel with the samples to be tested At least until the DNA isolation technique becomes routine, those extracts yielding negative results should be tested to confirm that the DNA can serve as a suitable PCR substrate This is easily done by amplifying a fragment from an endogenous single-copy gene (e g , cytosolic triose phosphate isomerase) (*see* **Fig. 1**).

7. For blot analysis, we normally load 2 μg of DNA per gel lane, although higher amounts can be used Routinely, DNA aliquots are run undigested to confirm that the introduced sequences comigrate with rice genomic DNA, indicating integration in the genome. Additional aliquots are digested with ~8 U of restriction enzyme for 4 h in a total volume of 20–30 μL After adding loading buffer, the entire reaction can then be loaded in a single well of a gel Positive and negative controls should always be included As a negative control, we use wild-type T309 DNA digested with the same restriction enzyme as the test samples A one copy/haploid genome reconstruction is included as a positive control This consists of 2 μg of wild-type rice DNA that has been spiked with plasmid DNA. The amount of plasmid to add can be determined by the following equation

$$n*D*P*660 / K*6.02 \times 10^{23} = M$$

where $n$ = the number of gene copies desired in the reconstruction, $D$ = μg genomic DNA loaded per lane; $P$ = plasmid size in bp, $K$ = 0 6 × $10^{-12}$ μg, the mass of a haploid copy of the rice genome, and $M$ = μg of plasmid DNA to use

The reconstruction and putative transgenic rice DNAs should be digested with an enzyme(s) to release a DNA fragment that would indicate whether the introduced gene is intact When compared to the intensity of the reconstruction, the intensity of this fragment can also be used to estimate the copy number of the introduced gene Additional copy number information can be obtained by digesting the DNA with an enzyme that cuts once in the center of the introduced gene If the probe hybridizes only to the sequences on one side of the site, the minimum copy number of the introduced gene will equal the number of hybridizing fragments The copy number would be half that number if the hybridization probe is complementary to sequences on both sides of the restriction site After blotting and hybridization with a high specific activity probe (~1 × $10^9$ cpm/μg DNA), single copy/haploid genome fragments can be visualized by overnight exposure (–70°C, one enhancing screen) to Kodak X-OMAT XAR-5 film, or 2 h exposure to a phosphoimager plate

8. Cover the lower portion of the gel with a folded paper towel to avoid nicking the low-mol-wt DNA (~6 kb and less). Expose the uncovered portion of the gel to a UV light (254 n*M*) The time of exposure and distance from the light source must be determined empirically to optimize DNA transfer, but allow efficient hybridization once blotted (Presumably, thymidine dimers or other products produced during UV exposure interfere with hybridization ) Begin with a 15-s exposure at ~10–15 cm For this step, we use a Stratagene UV crosslinker (3000 μW/cm$^2$) and expose for 0 6 min The upper 2 cm of the gel should be exposed for another 0.4 min to further nick the uncut genomic DNA
9. Binding of radiolabeled probe to contaminants (body oils, proteins, and so on) on the membrane can lead to high background. Therefore, care should be taken when handling the membranes Always use gloves or clean, blunt forceps, and avoid contacting dirty surfaces

10 It is essential that bubbles are not present to assure uniform flow of buffer, and thereby uniform transfer of the DNA, to the membrane
11 Parafilm or other hydrophobic material can be butted up to the gel on all sides to prevent overhanging paper from absorbing buffer without it going through the gel
12 Use $5 \times 10^6$ cpm denatured probe/mL hybridization solution. To denature, dilute the volume of probe needed to at least 50 µL with $H_2O$, heat denature (95–100°C, 5 min), and fast chill for 2–3 min on wet ice prior to adding to the hybridization solution.
13 If the membrane becomes dry, it is virtually impossible to remove the hybridized probe. If kept moist, ~90–100% of the probe can be removed and the blot reused (We have reused nitrocellulose blots 6–7 times with only limited loss in sensitivity.) Strip the probe by incubating it in 200–300 mL of 30 m*M* NaOH, 1 m*M* EDTA for 15 min at room temperature, with gentle agitation Decant and neutralize with a similar volume of Southern neutralization solution Use a radiation monitor before and after stripping to evaluate the effectiveness of probe removal If some radioactivity remains, expose the blot for a specific time to determine the background level To re-use the blot, the prehybridization step can be omitted Add the hybridization solution (without probe) and incubate at 65°C for 1–2 h before adding the probe

## Acknowledgments

Support from the Rockefeller Foundation Rice Biotechnology Program, the Texas A&M Office of Sponsored Projects, TAES Research Enhancement Program, and the Texas Advanced Technology Program (Projects 010366-108 and 010366-149) is gratefully acknowledged. We thank Kimberly Snowden for critical reading of this manuscript.

## References

1 Southern, E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98,** 503–511
2 Taylor, B. H , Manhart, J R., and Amasino, R M (1993) Isolation and characterization of plant DNAs, in *Methods in Plant Molecular Biology and Biotechnology* (Glick, B R and Thompson, J E , eds ), CRC, Boca Raton, pp 360
3. Agudo, L. d C., Gavidia, I., Perez-Bermudez, P., and Sugura, J (1995) PEG precipitation, a required step for PCRF amplification of DNA from wild plants of *Digitalis obscura* L *Biotechniques* **18,** 766–768.
4. Xu, Y , Buchholz, W G , DeRose, R T , and Hall, T C (1995) Characterization of a rice gene family encoding root-specific proteins *Plant Mol Biol* **27,** 237–248
5. Matzke, M A. and Matzke, A. J M. (1995) How and why do plants inactivate homologous (trans)genes? *Plant Physiol* **107,** 679–685.
6 Finnegan, J and McElroy, D (1994) Transgene inactivation· plants fight back! *Biotechnology* **12,** 883–888

7 Flavell, R. B (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication *Proc Natl Acad Sci USA* **91,** 3490–3496
8 Chamberlin, M A and Bowen, B (1995) Nuclear changes in maize cells transformed by DNA-coated microprojectiles, in *24th Annual Keystone Symposia*, Journal of Cellular Biochemistry, Hilton Head Island, SC, pp 138
9 Hall, T C , Xu, Y , Huntley, C C , Yu, H , Seay, J , Connell, J C , Lepetit, M , Dong, J , Wallace, D , Way, M O., and Buchholz, W G (1993) Transgene expression and agronomic improvement of rice *Phil Trans R Soc Lond B* **342,** 197–201
10 Chalfie, M , Tu, Y , Euskirchen, G , Ward, W W , and Prasher, D G (1994) Green fluorescent protein as a marker for gene expression *Science* **263,** 802–805.
11 Xu, Y , Harris-Haller, L W , McCollum, J C , Hardin, S H , and Hall, T C (1993) Nuclear gene encoding cytosolic triosephosphate isomerase from rice (*Oryza sativa* L ) *Plant Physiol* **102,** 697

# 42

# PCR Analysis of Transgenic Tobacco Plants

**Dawn Worrall**

## 1. Introduction

Although the display of antibiotic resistance can be a good indication that a regenerated plant is transformed with introduced DNA, escapes that represent partial or no transfer may be present within the population of primary transformants. The polymerase chain reaction (PCR) provides us with a technique for rapidly analyzing large numbers of putative transformants for the presence of a transgene DNA sequence.

Frequently, plant DNA extraction protocols are time-consuming and not applicable to very small amounts of tissue. The use of PCR to analyze large numbers of samples has therefore been unfeasible. However, since the publication of a small-scale DNA extraction method by Edwards et al. *(1)*, screening of transgenic plants by PCR has become a routine procedure. The method includes few steps and does not involve the use of phenol or chloroform. Many samples can therefore be processed in a short period of time without exposure to toxic chemicals.

PCR can provide us with a useful tool to demonstrate the presence of specific DNA sequences within the genome; however, care must be taken to include the appropriate control PCR experiments. When *Agrobacterium* has been used as the transformation vector, it is important to be able to distinguish between *Agrobacterium* contamination and genuine PCR positive results. The presence of *Agrobacterium* can be tested by carrying out a control PCR experiment, which involves a primer that is specific to the binary vector sequence from beyond the classically defined T-DNA border region. Included in **Table 1** is the sequence of a non–T-DNA right border (RB) primer *(2)* designed against the pBin19 binary vector *(3)*. This primer can be used in conjunction with *npt*-II 5' primer (also shown in **Table 1**), if the binary vector was

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

**Table 1**
**Primer Sequences for some Commonly Used Promoters and Reporter Genes**

| Gene/promoter | Primer sequences | Size of PCR fragment, bp |
|---|---|---|
| *npt*-II gene | 5' TCT CAC CTT GCT CCT GCC 3' (forward primer)<br>5' AGG CGA TAG AAG GCG ATG C 3' (reverse primer) | 464 |
| *gus*A gene | 5' AGC ATC TCT TCA GCG TAA GG 3' (forward primer)<br>5' TGA ACA ACG AAC TGA ACT GG 3' (reverse primer) | 611 |
| *nos* promoter | 5' GAC AAG CCG TTT TAC CGT TTG GAA CTG 3' (forward primer)<br>5' CTG CAG ATT ATT TGG ATT GAG AGT G 3' (reverse primer) | 270 |
| CaMV35S promoter | 5' CTA CTC CAA AAA TGT CAA AGA TAC AGT C 3' (forward primer<br>5' GGG CTG TCC TCT CCA AAT G 3' (reverse primer) | 370[a] |
| non-tDNA RB[b] | 5' CGC TCT TTT CTC TTA GGT TTA 3' (forward primer) | |
| *npt*-II 5' | 5' GTC ATA GCC GAA TAG CCT C 3' (reverse primer) | |

[a]Expected size for a single 35S promoter If a double 35S promoter is present within the construct, two PCR products will be obtained, one of 370 bp and the other 775 bp

[b]This primer can be used in conjunction with the *npt*-II 5' primer or a construct-specific primer to check for *Agrobacterium* contamination

based on pBin19, or with a construct-specific primer if a second vector primer is not available.

Recently, Martineau et al *(4)* have examined *Agrobacterium*-transferred DNA in a population of several hundred independent transformed plants representing several crop species From this study it appears that DNA from beyond the border-repeats in the original bacterial plasmid are also integrated into the genomes of 20–30% of the transgenic plants. This high frequency of beyond-the-border DNA transfer has several implications. First, the use of a primer that is designed against DNA sequences flanking the borders for identifying *Agrobacterium* contamination on primary transformants might not be as clear cut as expected. Second, researchers using T-DNA tagging as a means of cloning genes may encounter problems when attempting to identify adjacent plant DNA sequences because the T-DNA is larger than expected.

Despite these findings, if care is taken when devising PCR experiments, the results can be informative. In the PCR experiment shown in **Fig. 1**, the primers were designed to identify an introduced β(1,3)-glucanase transgene *(5)*. However, the native β(1,3)-glucanase gene is also recognized, but this gene can be distinguished from the transgene by the presence of an intron that increases the mol wt of the amplified product. The PCR amplification of the native β(1,3)-glucanase gene also served as a convenient positive control for each sample

The PCR method presented here has some negative points, but it can be extremely useful for the preliminary screen of large numbers of regenerated putative transformed plants. However, it is important to remember that much more information about the number of copies and arrangement of the introduced DNA within the plant genome can be obtained by carrying out Southern analysis (*see* Chapters 41 and 43)

## 2. Materials

### 2.1. DNA Extraction

1. Disposable grinders/spatulas (Sarstedt, cat no 81970)
2. PCR DNA extraction buffer. 200 m*M*Tris-HCl, pH 7 5, 250 m*M*NaCl, 25 m*M* EDTA, 0 5% SDS. Store at room temperature
3. Propan-2-ol.
4. TE. 10 m*M* Tris-HCl, pH 7 5, 0 1 m*M* EDTA.

### 2.2. PCR from Plant DNA

1. *Taq* DNA polymerase (Promega, Southampton, UK)
2. PCR buffer (10X) Supplied with the *Taq* DNA polymerase. Store at –20°C
3. dNTP stock 2 m*M* of each dNTP Store at –20°C
4. Thermal cycling machine (Cetus, Warrington, UK)

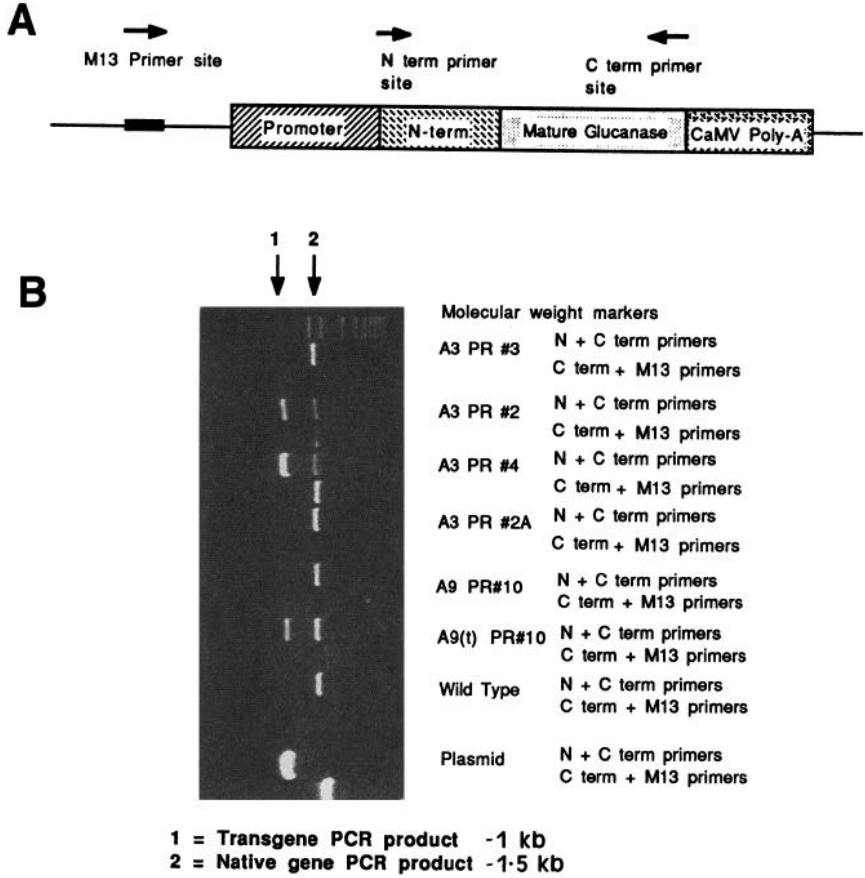



Fig. 1. PCR analysis to confirm the presence of an introduced modified PR β(1,3)-glucanase gene in regenerated tobacco plants. **(A)** Schematic diagram to illustrate the position of primer sites on the tDNA. **(B)** PCR products separated on a 0.8% agarose gel. In this example, the presence of a native β(1,3)-glucanase gene within the tobacco DNA served as a positive control for the PCR reaction. The native gene contains an intron of approx 500 bp, so it is readily distinguishable from the transgene. This example shows that some of the regenerated plants did not contain the introduced β(1,3)-glucanase gene (e.g., samples A9 PR #10 and A3 PR #3), and therefore represented escapes.

β(1,3)-glucanase primer sequences:
Forward primer 5' GGGTCTAGACCATGGCTGCTATCACACTCCTAGG 3'
Reverse primer 5' GGGCCGCGGTCACCCAAAGTTGATATTATATTTGG 3'

## 2.3. Analysis of PCR Products

1. High gelling temperature agarose (molecular biology grade, e.g., Seakem™, Flowgen, Lichfield, UK).
2. Electrophoresis buffer (10X TAE): 4*M* Tris-acetate, pH 8.0, 10 m*M* EDTA.

3 DNA loading buffer (10X) 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% (w/v) Ficoll (type 400).
4. Ethidium bromide solution. 5 mg/mL ethidium bromide dissolved in water (**Caution:** Ethidium bromide is a mutagen and irritant)
5 DNA size markers· 1-kb ladder (BRL, Paisley, UK)
6 Electrophoresis apparatus (e g., Bio-Rad, Hemel, Hempstead, UK)
7 UV transilluminator plus orange G filter.

## 3. Methods

### 3.1. DNA Extraction

1. Samples for PCR analysis can be taken from the plant by pinching out a disk of material (usually leaf) using the lid of an Eppendorf (*see* **Note 1**)
2 Grind up the plant tissue, using a disposable grinder, for approx 15 s
3. Add 400 µL PCR DNA extraction buffer and vortex for 15 s
4. Leave at room temperature until all of the samples have been processed (*see* **Note 2**)
5 Centrifuge at 15,000*g* in a microcentrifuge for 1 min
6 Remove 300 µL of supernatant to a fresh tube and add an equal volume of propan-2-ol
7 Incubate at room temperature for 2 min (*see* **Note 3**), before centrifuging at 15,000*g* for 10 min
8. Completely remove supernatant and vacuum- or air-dry the pellet (*see* **Note 4**)
9 Resuspend the pellet in 100 µL TE (*see* **Note 5**)
10 Typically, 2 µL of this DNA solution can be used for PCR analysis.

### 3.2. PCR from Plant DNA

1. For each DNA sample, set up a PCR reaction A typical reaction 2 µL DNA solution (*see* **Note 6**), 2 µL 10X PCR buffer (*see* **Note 7**), 0 1 m*M* dNTPs, 1 5 U *Taq* DNA polymerase, 1 µ*M* of each primer, make volume of reaction up to 20 µL with sterile water (*see* **Note 8**)
2 Overlay the reactions with a drop of mineral oil
3 Carry out 30 cycles of denaturation (95°C for 1 min), annealing (50–60°C [*see* **Note 9**] for 1 min), and extension (72°C for 1 min/kb of predicted DNA product)
4. Check the PCR products by electrophoresis on an agarose gel.

### 3.3. Detection and Analysis of PCR Products

1 Prepare a 1% agarose gel in 1X TAE buffer. Before pouring into casting tray, add ethidium bromide solution (to a final concentration 0 05 mg/mL).
2. Add 0.1 vol DNA loading buffer to the PCR reaction through the oil
3 Place the gel in electrophoresis apparatus, and cover with 1X TAE buffer.
4 Add ethidium bromide solution to the 1X TAE buffer (to a final concentration of 0 05 mg/mL)

5 Load the PCR samples into the gel and also include in one well approx 1 μg, 1-kb ladder mol-wt markers.
6 Run the gel at 60–100 V until the bromophenol blue visual dye marker has migrated to approx 4 cm from the bottom of the gel
7 Visualize the PCR products on a UV transilluminator and photograph using Polaroid Land Camera or video camera (Flowgen) with an orange G filter to maximize the contrast (*see* **Note 10**)

## 4. Notes

### 4.1. DNA Extraction

1 If the plant material is to be processed directly, store at room temperature Alternatively, store on dry ice or at –80°C Leaf disks are the usual source of material for this extraction procedure However, the technique has also proved to be successful with small amounts of tissue from hairy root cultures
2 Samples do not appear to deteriorate after 1 h of storage under these conditions
3 At this stage, a stringy DNA precipitate may be visible
4 DNA pellets are often green or brown in color, this is expected from a crude extraction procedure
5 The pellet may not resuspend completely, but the small amount of debris remaining does not appear to inhibit the PCR amplification

### 4.2. PCR from Plant DNA

6 Initially, it is advisable to try several different volumes of the sample DNA to determine the optimum
7 Although PCR buffer is usually supplied with *Taq* DNA polymerase, a recipe is provided below for those who wish to assemble their own reagents. It is useful to have a recipe, so that components can be varied, if neccessary For example, certain primer sets work more efficiently with different magnesium concentrations 1X PCR buffer· 44 m*M* Tris-HCl, pH 8.8, 11 m*M* $NH_4SO_4$, 4 5 m*M* $MgCl_2$, 7 m*M* β-mercaptoethanol, 113 μg/mL BSA, 4 5 m*M* EDTA
8 To speed up the process and to minimize errors, make a stock solution containing enough buffer, primers, *Taq* DNA polymerase, dNTPs, and water for all the reactions, then aliquot into the PCR tubes before adding the DNA sample. Adding the DNA template last minimizes the risk of contamination between samples It is generally good practice to include a water negative control and a plasmid positive control (1 ng plasmid DNA is sufficient) among the plant DNA samples
9 The annealing temperature can be determined from the sequence of the primers The melting temperature for duplex DNA of less than 20 bp can be calculated as follows. 2°C for every AT pair, plus 4°C for every GC pair. To obtain the annealing temperature, 5°C is subtracted from the melting temperature

### 4.3. Detection and Analysis of PCR Products

10 The product should appear as a sharp band at the expected size Small DNA products (primer dimers) are sometimes visible as diffuse bands close to the leading

front of the gel Additional faint bands, which may be caused by nonspecific priming, may also appear

## Acknowledgments

I would like to thank Diane Hird for supplying the primer sequences for the *nos* and *35S* promoters, Hayley McArdle for the *gus* and *npt*-II gene primer sequences, and Leonie Rooke for the *npt*-II 5' primer sequence.

## References

1 Edwards, K , Johnstone, C , and Thompson, C (1991) A simple and rapid method for the preparation of genomic DNA for PCR analysis. *Nucleic Acids Res* **19,** 1349

2 Spychalla, J. P and Bevan, M W. (1993) Agrobacterium mediated transformation of potato stem and tuber tissue, regeneration and PCR screening for transformation, in *Plant Tissue Culture Manual· Fundamentals and Applications* BII. Kluwer Academic, Dordrecht, pp. 1–18

3 Bevan, M. W. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* **12,** 8711–8721

4 Martineau, B , Voelker, T A , and Sanders, R A (1994) On defining T-DNA. *Plant Cell* **6,** 1032–1033

5 Worrall, D., Hird, D L , Hodge, R , Paul, W., Draper, J., and Scott, R. (1992) Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco *Plant Cell* **4,** 759–771

# 43

# Southern Analysis of Transgenic Tobacco Plants

**Dawn Worrall**

## 1. Introduction

Southern analysis *(1)* is routinely carried out to determine whether a plant regenerated from tissue-culture has been transformed with foreign DNA. The technique of Southern analysis begins with the extraction of genomic DNA from the plant, digestion of the DNA with diagnostic-restriction enzymes, and fractionation of the restricted DNA by agarose gel electrophoresis Following the transfer of the fractionated DNA to a nylon membrane by capillary blotting (Southern blotting), a radioactively labeled fragment of the foreign DNA is used for the detection of homologous sequences within the plant genomic DNA. This technique allows not only the detection of foreign DNA, but also an estimation of the number of copies and the arrangement of the foreign gene(s) within the plant genome.

Polymerase chain reaction (PCR) techniques can also be used to demonstrate the presence of foreign DNA (*see* Chapters 41 and 42). However, although PCR gives results rapidly, it is necessary to carry out a range of control experiments to avoid false-positive results that may arise from contaminating DNA sequences. The PCR method can be useful when there are only limited amounts of tissue available for analysis. However, if there is sufficient material, Southern analysis should be performed in preference since much more information can be gained

Detailed protocols are presented below for the extraction of genomic DNA, Southern blotting, radioactive labeling of DNA fragments, and for hybridization. A method is also included for the stripping and reprobing of DNA blots.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

## 2. Materials

### 2.1. Genomic DNA Extraction

1 Liquid nitrogen
2 Pestles and mortars
3 50-mL Polypropylene tubes
4 2X cetyl triethylammonium bromide (CTAB) solution. 2% (w/v) CTAB, 100 m*M* Tris-HCl, pH 8.0, 1.4*M* NaCl, 20 m*M* EDTA.
5 Chloroform octanol. mix the two solutions in the ratio 24.1.
6 10% CTAB solution· 10% (w/v) CTAB, 0 7*M* NaCl
7 CTAB precipitation buffer 1% (w/v) CTAB, 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA
8 1*M* NaCl
9 Absolute ethanol
10 70% (v/v) Ethanol
11 TE. 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.2.
12 RNase A (Sigma, Poole, UK) Prepared by dissolving in sterile $dH_2O$ to a concentration of 10 mg/mL and heat-treating by boiling for 10 min to destroy any contaminating DNases. Store at –20°C.
13 Phenol.chloroform isoamyl alcohol solution Mix the solutions 25 24 1 The phenol can be purchased ready-equilibrated in Tris buffer from Sigma This solution can be stored in the dark at 4°C for up to 6 wk **Caution:** Phenol is toxic
14 5*M* NaCl

### 2.2. Southern Blotting

#### 2 2 1. Restriction Enzyme Digestion of Genomic DNA and Agarose Gel Electrophoresis

1. High-gelling temperature agarose (molecular biology grade, e g , Seakem™, Flowgen, Lichfield, UK)
2. Electrophoresis buffer (10X TAE) 4*M* Tris-acetate, pH 8 0, 10 m*M* EDTA
3. DNA loading buffer (10X) 0.25% (w/v) bromophenol blue, 0 25% (w/v) xylene cyanol, 25% (w/v) Ficoll (type 400)
4 Ethidium bromide solution 5 mg/mL ethidium bromide dissolved in water (**Caution:** Ethidium bromide is a mutagen and irritant). Store this solution at 4°C
5 DNA size markers· 1-kb ladder (BRL, Paisley, UK)
6 Electrophoresis apparatus (e g , Bio-Rad, Hemel, Hempstead, UK)
7 UV transilluminator (e.g , Flowgen) and an orange G filter

#### 2.2 2. Preparation of Gel for DNA Transfer

1 Depurinating solution· 0 25*M* HCl
2 Denaturing solution. 0 5*M* NaOH, 1.5*M* NaCl
3. Neutralizing solution. 3.0*M* NaCl, 0 5*M* Tris-HCl, pH 7.4.

### *2.2.3. DNA Transfer*

1. Nylon membrane (e g., Zeta Probe GT, Bio-Rad).
2. 10X SSC: 1.5*M* NaCl, 0 15*M* trisodium citrate, pH 7 0.
3. 3MM paper (Whatman, Maidstone, UK).
4. Paper towels
5. Sponges
6. Plastic tray.
7. Clingfilm/Sealing film (Whatman).
8. 80°C oven

## *2.3. Radiolabeling a DNA Fragment by the Random Primer Method*

1. GeneClean II™ DNA purification kit (Bio 101, supplied by Anachem, Luton, UK)
2. Solution O: 1.25*M* Tris-HCl, 0 125*M* $MgCl_2$, pH 8 0 (stored at 4°C).
3. Solution A: 1 mL solution O + 18 µL β-mercaptoethanol + 5 µL of each dATP, dTTP, and dGTP (each triphosphate previously dissolved in TE, pH 7 0, at a concentration of 0.1 *M*; stored at –20°C).
4. Solution B. 2*M* HEPES, titrated to pH 6 6 with 4*M* NaOH (stored at 4°C)
5. Solution C: Hexadeoxyribonucleotides (Pharmacia, St. Albans, UK) dissolved in TE at a concentration of 90 $OD_{260}$ U/mL (stored at –20°C)
6. Oligolabeling buffer (OLB): Prepared by mixing together solutions A, B, and C in a ratio of 100:250:150, respectively. Store in aliquots at –20°C This protocol assumes that the radiolabel is α[$^{32}$P]dCTP.
7. Sterile $dH_2O$.
8. BSA (10 mg/mL).
9. α[$^{32}$P]dCTP (10–50 µCi)
10. Klenow fragment of *Escherichia coli* DNA polymerase I
11. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.2.
12. Sephadex G-50 column, equilibrated in TE, pH 7 2.
13. Liquid scintillant: 5% (w/v) POP in toluene
14. Scintillation counter.

## *2.4. Hybridization of a DNA Probe to a Southern Blot*

1. Church buffer: 0.25*M* $Na_2HPO_4$, pH 7.2, 7% SDS.
2. Radioactive probe
3. Wash I: 20 m*M* $Na_2HPO_4$, pH 7.2, 5% SDS.
4. Wash II: 20 m*M* $Na_2HPO_4$, pH 7.2, 1% SDS
5. Saran Wrap (Dow, supplied by SLS, Wilford, UK).
6. X-ray film (e g., Amersham, Little Chalfont, UK, Hyperfilm-MP)
7. Hybaid (Teddington, UK) oven and glass bottles
8. Geiger counter
9. Autoradiography cassette containing signal intensifying screens (e.g , from GRI, Dunmow, UK).

## 3. Methods

### 3.1. Genomic DNA Extraction

One of the major limiting factors in the isolation of plant DNA is the efficient disruption of the cell wall. There is often a compromise between complete wall breakdown and the length and yield of DNA. Another problem often encountered with plant DNA isolation is the presence of contaminating carbohydrates and phenolics. These compounds are difficult to separate from the DNA and can interfere with the quantification of nucleic acids by spectrophotometry. More importantly, these contaminants also inhibit the activity of DNA modifying enzymes. To try to reduce this contamination problem, plant tissue that is low in such compounds should be used as source material. Young, fully expanded leaves are often suitable for this purpose

In the method described below, which is based on that of Murray and Thompson *(2)*, the plant cell wall is disrupted by grinding the frozen plant tissue with a mortar and pestle. The ground tissue is then incubated in an aqueous solution containing chelating agents, to inhibit nuclease action, and a detergent, to solubulize membranous material The success of the method is because of the fact that in the presence of high salt concentrations, the CTAB detergent binds nucleic acid to form soluble complexes. If the salt concentration is subsequently decreased, nucleic acid–CTAB complexes precipitate, but contaminating carbohydrates are left behind in solution. The resulting nucleic acids are therefore relatively free from compounds that can contaminate DNA preparations.

This protocol has routinely been used to extract DNA from tobacco leaves and whole mature *Arabidopsis* plants. A yield of up to 500 µg of genomic DNA can be expected from 5 g tobacco leaf material. A review of nucleic acid extraction protocols for different species of plants can be found in **ref. *3***.

1 Grind 5 g tobacco leaf material (*see* **Note 1**) in liquid nitrogen, in a precooled pestle and mortar
2 Transfer the frozen powder to a 50-mL tube.
3 Add 1–2 mL per gram of 2X CTAB buffer preheated to 95°C. Mix thoroughly by gentle repeated inversion of the tube and transfer to a 56°C water bath (*see* **Note 2**)
4 Incubate for 20–60 min, mixing occasionally
5 Allow the tubes to cool, and add an equal volume of chloroform octanol
6 Emulsify by gentle shaking and centrifuge for 10 min at 1000*g* (*see* **Note 3**)
7 Transfer the upper aqueous phase, which contains the DNA, to a fresh tube and add 0.1 vol 10% CTAB solution preheated in a 56°C water bath
8. Mix thoroughly, until the solution clears, and repeat chloroform:octanol extraction and centrifugation
9 To the new aqueous phase, add an equal volume of CTAB precipitation buffer, mix thoroughly, and allow to stand at room temperature for at least 20 min (*see* **Note 4**)

10. Spin down precipitate at 1000*g* for 15 min
11 Drain tubes well and resuspend the pellet in 1*M* NaCl (1 mL/g starting material) (*see* **Note 5**)
12 Add 2X vol of ethanol to precipitate nucleic acids Store at –20°C for 30 min Spin down nucleic acids and rinse the pellet twice with 70% ethanol (*see* **Note 6**)
13 Finally spin down nucleic acids for 5 min at 1000*g*, remove all 70% ethanol, and vacuum-dry pellets for 5 min
14 Resuspend the pellet in TE (100 µL/g of starting material) (*see* **Note 7**)
15. If RNA-free DNA is required, **steps 16–20** can be followed (*see* **Note 8**).
16 Add 10 µL of RNase stock to the solution and incubate at 37°C for 30 min
17. Add an equal volume of phenol chloroform isoamyl alcohol and emulsify by inversion Centrifuge at 15,000*g* in a microcentrifuge for 2 min.
18. Remove the aqueous phase to a fresh Eppendorf tube and add 0 05 vol 5*M* NaCl and 2 vol of ethanol Store at –20°C for 30 min
19 Spin down nucleic acids and rinse the pellet with 70% ethanol
20. Remove all 70% ethanol and vacuum-dry pellet for 5 min, before resuspending in TE
21 Analyze the DNA by fractionating 1 µL on a 1% agarose gel

## 3.2. Southern Blotting

### 3.2.1. Restriction Enzyme Digestion of Genomic DNA and Agarose Gel Electrophoresis

It is important to obtain as much information as possible on the structure and organization of transferred genes within the plant genome. The number of copies of T-DNA, whether they are tandemly linked or dispersed, and the position within the genome can have a great effect on the expression levels and inheritance patterns of introduced genes

By selecting appropriate restriction enzymes to cut the plant genomic DNA, it is possible to gain information about the organization of the T-DNA within a particular transformant. The number of T-DNA copies can be estimated by two commonly used methods. The first method involves digesting genomic DNA with restriction enzymes that cut twice within the target T-DNA, to yield an internal fragment. The copy number is estimated by calibrating the intensity (following autoradiography) of the internal fragment band against samples of known copy numbers of the foreign DNA (usually plasmid DNA) run on the gel. Genomic DNA from untransformed plants must be added to the plasmid copy number control samples, to provide a similar background to the transgenic sample for hybridization. In the second method, the genomic DNA is digested with a restriction enzyme that cuts once in the target DNA. Here, border fragments are generated, where the second site of cleavage is in the genomic DNA. Since the site of the second cleavage is expected to be different for each independent insertion event, the number of bands observed on the

autoradiogram indicates the number of copies present Tandemly linked T-DNAs can also be diagnosed by cutting with restriction enzymes that cut once within the construct. In this case, a single intense band is generated from internal fragments within the tandem repeat Border fragments of different sizes are also generated. This experiment is important in order to differentiate between single and multiple insertion events when segregation data have indicated a single locus insertion In all cases it is important to run a negative control using nontransformed genomic DNA, to check that, under the experimental conditions used, there is no background hybridization that could confuse the analysis.

1 Digest genomic DNA with appropriate restriction enzymes (*see* **Note 9**)
2 Prepare a 0.8% agarose gel in 1X TAE buffer. Before pouring into casting tray, add ethidium bromide solution (to a final concentration 0.05 mg/mL)
3. Mix digested DNA with a 0 1 vol gel loading buffer.
4 Place the gel in electrophoresis apparatus, and cover with 1X TAE buffer
5. Add ethidium bromide solution to the 1X TAE buffer (to a final concentration of 0 05 mg/mL)
6 Load the DNA samples and 1 μg 1-kb ladder on the gel
7 Run the gel at 60–100 V until the bromophenol blue visual dye marker has migrated approx three-fourths of the way down the gel.
8 Visualize the DNA on a UV transilluminator and photograph, using Polaroid Land Camera or video camera (Flowgen) with an orange G filter to maximize the contrast (*see* **Note 10**)

### 3.2.2. Preparation of Gel for DNA Transfer

1 After photographing, incubate the agarose gel in depurinating solution for 15 min at room temperature, with gentle agitation This acts to depurinate or acid-nick the DNA, and increases the efficiency of DNA transfer to the membrane
2 Remove the depurinating solution and rinse the gel in distilled water
3. Incubate the gel in denaturing solution for 30 min at room temperature, with gentle agitation (*see* **Note 11**)
4. Rinse the gel in distilled water
5 Incubate the gel in neutralizing solution for 30 min at room temperature, with gentle agitation (*see* **Note 12**)
6 The gel is now ready for DNA transfer

### 3.2.3. DNA Transfer

There are many different membranes commercially available for nucleic acid blotting The nylon-based ones are the most practical, because they are very strong and can withstand repeated use (which is important if the membranes are to be probed sequentially, such as with more than one DNA sequence), with minimal loss of sensitivity The products that we have used in our laboratory are Zeta-Probe GT (Bio-Rad), Gene Screen Plus (Dupont,

Hounslow, UK), and Hybond N (Amersham). For ease of use, high signal-to-noise ratio, and durability, we have found Zeta-Probe GT to be the most suitable. It is important to follow the manufacturer's guidelines for each type of membrane used. However, the methods for use are all broadly similar, and the protocols given below are suitable for all three membranes mentioned above.

1. Cut the nylon membrane to the same size as the gel and presoak the membrane in either distilled water or the transfer buffer (10X SSC), according to the membrane manufacturer's instructions
2. Cut three pieces of 3MM paper to the same size as the gel, and soak in the transfer buffer
3. Lay sponges to a thickness of 6–8 cm in a plastic tray and flood with 10X SSC
4. Lay two pieces of 3MM paper, larger than the gel, on the sponges (*see* **Note 13**).
5. Lay the gel on top of the 3MM paper, checking that there are no air bubbles Cover the remaining surface of the 3MM and sponges with cling film (*see* **Note 14**)
6. Carefully place the membrane on the gel, ensuring that there are no air bubbles present (*see* **Note 15**)
7. Place the presoaked pieces of 3MM paper on top of the gel, followed by a stack (approx 10 cm thick) of paper towels Place a glass plate and a suitable weight (e.g , a 500-mL medical flat) on top of the stack and leave standing overnight to allow DNA transfer to take place
8. If necessary, top up the transfer buffer in the tray before leaving the blot overnight
9. Remove the membrane, wash it briefly in 10X SSC, and then leave it to air-dry on 3MM paper, DNA-side up
10. Follow the manufacturer's instructions to irreversibly crosslink the DNA to the membrane (*see* **Note 16**) The membrane is now ready for hybridization

### 3.3. Radiolabeling a DNA Fragment by Random Primer Method

A method for radiolabeling DNA fragments in agarose gel slices to high specific activity was devised by Feinberg and Vogelstein *(4)*. However, the specific activity of the DNA fragments can be significantly increased by purification of the DNA from the agarose gel, by electroelution or by using purification kits (e.g., GeneClean II DNA purification kit available from Bio 101). To estimate the amount of DNA that should be labeled, the hybridization membrane manufacturer's guidelines should be consulted, typically, the probe should be at a concentration of 10–50 ng/20 mL of hybridization solution. It is also important to generate a probe with a good specific activity, to produce a high signal-to-noise ratio during hybridization. A specific activity of 108 cpm/μg probe is generally recommended

The equipment available for carrying out hybridizations has improved over recent years, allowing the reactions to be carried more safely and with greater ease. The rotisserie-type hybridization oven (e.g., Hybaid) can be very useful. Multiple hybridization reactions can be carried out simultaneously (provided the same temperature is required), and the glass bottles used in the oven minimize the risks associated with the handling of radioactively labeled solutions and membranes.

The protocol presented here is designed to allow the detection of a specific DNA fragment using a homologous, radioactively labeled DNA sequence as probe. Both the hybridization reaction and washes are carried out under stringent conditions, at 65°C, to minimize nonspecific hybridization. There are various hybridization buffers that can be used, but Church buffer *(5)* seems to produce the best results. The SDS within the buffer acts as a blocking agent, which, in conjunction with the reaction temperature of 65°C, eliminates problems of background hybridization. This buffer can be used successfully with any of the membranes mentioned above It is convenient to make up 2–3 L of both the hybridization buffer and the wash solutions and store them at 65°C.

1 Purify the DNA fragment by electroelution or using the Gene Clean kit (Bio 101), and resuspend in distilled water at a concentration of 10–100 ng/µL This stock can be stored for several months at –20°C
2 To a sterile Eppendorf tube, add 0 6 µL of BSA, 3 µL of OLB, 10–50 µCi [$^{32}$P]dCTP, and 0 6 µL (0 5 U) of the large fragment of DNA polymerase I (Klenow fragment) Store on ice
3 The final volume of the oligolabeling reaction will be 15 µL Therefore, add an appropriate volume of distilled water to 10–50 ng of the DNA probe stock solution and boil for 5 min to denature the DNA Centrifuge briefly to collect the contents to the bottom of the tube, and cool on ice before adding to the reaction mix.
4 Incubate the reaction at 37°C for approx 1 h (*see* **Note 17**)
5 Add 200 µL TE, pH 7 2, to stop the reaction
6 Fractionate the probe to remove the unincorporated [$^{32}$P]dCTP by running the reaction mixture through a Sephadex G-50 column, equilibrated in TE, pH 7 2 (*see* **Note 18**)
7 Pool the labeled fractions
8 Denature the probe by boiling for 5 min (*see* **Note 19**) immediately prior to adding to the hybridization solution (*see* **Subheading 3.4.**)

## 3.4. Hybridization of DNA Probe to Southern Blot

1 Place the membrane in a Hybaid glass bottle and add enough Church buffer to cover the membrane (typically, ca 10–25 mL) Prehybridize the membrane at

65°C for 15–30 min in a Hybaid oven (*see* **Note 20**) Occasionally, it may be necessary to alter the hybridization conditions (*see* **Note 21**).

2. Add the denatured radioactive probe to some fresh Church buffer (10 mL) and mix carefully.
3. Pour away the prehybridization buffer and replace it with the probe solution The hybridization should be carried out at 65°C, with constant agitation, for at least 12 h
4. Remove the probe solution (*see* **Note 22**) and replace with Wash I. Incubate at 65°C, with constant agitation, for 30 min. Remove the wash and repeat
5. Remove Wash I and repeat the procedure with Wash II
6. If the activity is not localized and the background is still high, repeat **step 5**
7. Remove excess wash solution, wrap the membrane in Saran Wrap, and expose to X-ray film at –80°C, in a cassette containing signal intensifying screens.
8. If desired, after autoradiography, the blot can be stripped and reprobed by following the protocol in the next section

### 3.5. Probe Stripping and Rehybridization

1. If reprobing is desired, do not let the membrane dry out between hybridizations (*see* **Note 23**)
2. Wash the membrane 2 × 20 min each in a large volume of 0 1X SSC/0 5% SDS at 95°C (*see* **Note 24**)
3. Check that all of the probe has been removed from the blot by exposing overnight

## 4. Notes

### 4.1. Genomic DNA Extraction

1. It is important not to crush the plant material or let it wilt, because this may affect the efficiency of the extraction The protocol can be successfully scaled up or down, depending on the amount of tissue available
2. After adding the 2X CTAB buffer to the ground plant material, the suspension is sometimes very viscous This is dependent on the source of the material, and the amount of polysaccharide present in the tissue If very viscous, it may be advantageous to dilute the extract further with aliquots of CTAB extraction buffer
3. CTAB will precipitate at temperatures below 15°C, so ensure that the centrifuge and rotor are at room temperature before inserting the tubes
4. This step precipitates CTAB–nucleic acid complexes by reducing the NaCl concentration; if no precipitate forms, add a little more precipitation buffer Precipitates may be stringy or fine suspensions The solution can be left for a longer time than indicated to allow precipitation, even overnight, as long as the temperature is maintained over 20°C
5. The CTAB–DNA pellet may not resuspend immediately, and it may be neccessary to heat the solution to 50°C It is often easier to resuspend the CTAB–DNA complexes if they have been pelleted in a low-speed benchtop centrifuge

6 The CTAB is soluble in ethanol, but the nucleic acids are not The ethanol washes remove any remaining CTAB from the nucleic acids, which may otherwise inhibit restriction-enzyme digestion
7 If highly pure DNA is required, the DNA can be purified on a CsCl/EtBr gradient at this stage
8 Instead of removing the RNA at this stage, RNase can be added to the DNA during restriction enzyme digestion In this case, 2 µL of 0 5 mg/mL RNase A (diluted from the 10 mg/mL stock, **Subheading 2.1.**) can be added to each reaction

## 4.2. Southern Blotting

### 4.2.1. Restriction Enzyme Digestion of Genomic DNA and Agarose Gel Electrophoresis

9. The amount of DNA digested varies with the source of the DNA Typically, 1–10 µg are digested to detect a single copy T-DNA, depending on the species For *Arabidopsis*, 500 ng–1 µg is sufficient, but for tobacco, 10 µg is usually required It is best to digest genomic DNA overnight
10 When photographing the gel, it is useful to put a ruler alongside to provide a reference for sizing the hybridizing bands later

### 4.2.2. Preparation of the Gel for DNA Transfer

11 The incubation period of 30 min is the minimum time required for denaturation, and can be increased to 2 h without any detrimental effects, providing high quality agarose has been used to make the gel
12 The incubation period of 30 min is the minimum time required for neutralization It is better to increase this incubation time, especially if nitrocellulose is being used, since this type of membrane becomes fragile if put in contact with a gel that is not completely neutralized

### 4.2 3. DNA Transfer

13. Ensure that the 3MM paper is saturated with buffer and that there are no air bubbles present, which would prevent local transfer of the DNA
14. Push the clingfilm 2 mm under the gel, this will prevent the buffer short circuiting the gel, which would result in poor transfer
15 Cut a corner off the membrane to allow orientation later, and mark the positions of the wells with a soft pencil
16 The DNA can be bound to the membrane by baking the membrane at 80°C for 30 min in a vacuum oven Alternatively, the DNA can be bound to the membrane by UV crosslinking Before using this method, the UV source has to be calibrated for the particular membrane ***(6)*** Automatically regulated UV crosslinking machines are now commercially available from several manufacturers (e g , Stratagene, Cambridge, UK)

### 4.3. Radiolabeling a DNA Fragment by the Random Primer Method

17 During the time that the oligolabeling reaction is proceding, set up prehybridization of the blot (*see* **Subheading 3.4.**)

18. This type of labeling reaction is usually very efficient, so the unincorporated [$^{32}$P]dCTP does not have to be separated from the probe DNA before adding to the hybridization solution However, genomic Southerns often require a long exposure time, so a clean background is necessary. In this case, probe fractionation can be beneficial

    When fractionating a probe for the first time, it is helpful to add blue dextran (10 mg/mL) to the reaction mixture just prior to fractionation. The dye co-migrates with the labeled DNA fragment, thereby facilitating identification of the desired fractions (the blue dextran does not interfere with the subsequent hybridization) It is now possible to buy ready made columns for DNA fractionation (e.g., Stratagene NucTrap purification columns)

19 It is important to boil the DNA probe thoroughly, because a double-stranded probe will not bind to sequences on the Southern blot

### 4.4. Hybridization of a DNA Probe to a Southern Blot

20. If a Hybaid oven is not available, the hybridization can be carried out in a plastic sandwich box with a tight-fitting lid, such as a Boehringer (Lewes, UK) box (cat no 800058). To prevent the membrane drying out during the hybridization period, a piece of Saran Wrap just bigger than the box should be floated on top of the hybridization solution Alternatively, a heat-sealed plastic bag can be used.

21 It is often useful to be able to alter the stringency (amount of nucleotide mismatch allowed) of the hybridization reaction and the washes With the Church buffer system, this can be acheived by altering the temperature of the hybridization and washes. Alternatively, the following buffer system can be used, in which the stringency can be altered by changing the salt concentration. Hybridization solution: 6X SSC, 10X Denhardts (50X Denhardts: 1% Ficoll [type 400], 1% PVP, 1% BSA), 0 5% SDS, 6% PEG 6000, 0 5 mg/mL sheared and denatured herring sperm DNA Wash solution I: 3X SSC, 0.5% SDS Wash solution II 0.5X SSC, 0.5% SDS.

22. The probe solution can be stored at –20°C and reused after boiling for 5 min

### 4.5. Probe Stripping and Rehybridization

23 The Zeta-Probe membrane should be stripped as soon as possible after autoradiography If this is not possible, ensure that the blot is well-wrapped with Saran Wrap and store in the freezer

24 A 1-L Duran bottle is useful for stripping the probe from Southern blots

## References

1 Southern, E M (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98,** 503–517
2 Murray, M. G and Thompson, W F (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8,** 4321–4325
3. Croy, E J , Ikemura, T , Shirsat, A., and Croy, R. R. D. (1993) Plant nucleic acids, in *Plant Molecular Biology Lab Fax* (Croy, R R D , ed ), Bios, Oxford, pp 21–47
4. Feinberg, A. P and Vogelstein, B. (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity *Anal Biochem* **137,** 266,267
5 Church, G M and Gilbert, W (1984) Genomic sequencing. *Proc Natl Acad Sci USA* **81,** 1991–1995
6 Scott, R. (1988) DNA restriction and hybridization, in *Plant Genetic Transformation and Gene Expression* (Draper, J., Scott, R , Armatage, P , and Walden, R , eds ), Blackwell, Oxford, pp 237–261

# 44

# Detection and Quantification of Transcript RNA in Transgenic Plants Using Digoxigenin-Labeled cDNA Probes

**Kara D. Webster and Hugh Barker**

## 1. Introduction

The detection of RNA transcript expressed from transgenes is often one of the first steps in the analysis of transgenic plants. Such analysis might include confirmation that RNA transcripts are of the expected size and the quantification of transcript in different transgenic lines. Until recently, the most common method of RNA detection involved the use of cDNA probes labeled with $^{32}P$ or $^{35}S$. However, the use of radioisotopes involves special procedures and facilities for handling and a requirement for the reliable delivery of radiochemicals, which frequently have short half-lives. Such facilities may be expensive and/or, in some countries, difficult to obtain. Furthermore, radioactive decay limits the time a probe can be used, $^{32}P$-labeled cDNA probes have a maximum useful lifespan of about 2 wk By comparison, nonradioactive probes do not have many of the problems associated with handling radioactive probes The digoxigenin (DIG) system developed by Boehringer Mannheim (Mannheim, Germany) for nonradioactive labeling of RNA and DNA probes has been developed so that sensitivity is comparable with radioactivity. This chapter will describe the use of DIG-labeled cDNA probes The use of RNA probes using the DIG system is described in Chapter 47.

The DIG system uses digoxigenin, a steroid hapten in the form of DIG-11-dUTP, to label DNA, RNA, or oligonucleotides. Probes can be produced by a number of methods. We will describe techniques for cDNA probes produced by polymerase chain reaction (PCR) and random priming. The process of hybridization and detection is relatively simple. Following a conventional Northern blotting procedure, labeled probes are hybridized to membrane-bound

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

nucleic acid, which are detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody and a chemiluminescent substrate. Chemiluminescent substrates can be visualized by exposure to X-ray film, thus providing a permanent record that is directly comparable to results that would be obtained using radioactive probes. Luminescent detection is fast and sensitive, and membranes can easily be stripped and reprobed

DIG probes have several advantages over radioactive probes. For example, using chemiluminescent substrates, detection can be completed with a 15- to 30-min exposure to X-ray film, compared to 1–3 d required for most $^{32}$P-labeled probes. Another advantage is that probes can be stored at 4°C or –20°C for many years with no apparent loss in sensitivity For example, radioactive and nonradioactive cDNA probes to tobacco rattle virus RNA were recently prepared from the same plasmid used to make a DIG probe nearly 3 yr earlier, and which had been stored at –20°C. In a comparative test, the stored DIG-labeled probe was as sensitive for detection as the freshly prepared DIG and radioactive probes. Many of the probe preparation, hybridization, and washing protocols are the same or similar for the DIG and radioactive systems. The following are techniques that we use for detection and quantification of RNA transcript in transgenic plants. The DIG system has many uses and variations, a more comprehensive guide and instructions can be found in **ref. *1***.

## 2. Materials

### 2.1. Boehringer Reagents

1 PCR DIG labeling kit This contains PCR buffer, 25 m*M* magnesium chloride, *Taq* DNA polymerase, and dNTP mixture containing DIG-11-dUTP.
2 DIG-High Prime (random priming labeling) kit Contains an optimized reaction mixture of buffer, random nucleotides, Klenow enzyme, dNTPs, and DIG-11-dUTP in 50% glycerol.
3 Positively charged nylon membrane
4. DIG-labeled control DNA
5 Blocking reagent
6 DIG Easy Hyb (hybridization) buffer
7. Anti-DIG-alkaline phosphatase, Fab fragments
8 CSPD (chemiluminescent) substrate.
9 DIG-labeled RNA mol-wt markers

### 2.2. Stock Solutions

Unless stated otherwise, autoclave and store at room temperature (*see* **Note 1**).

1 TE buffer 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0
2 Buffer 1. 100 m*M* maleic acid, 150 m*M* sodium chloride, pH 7.5, with NaOH.

3 Buffer 2. Blocking stock solution (*see* **item 5**) diluted 1 in 10 in buffer 1 This buffer should be made fresh every time it is used, because it deteriorates quickly
4 Buffer 3 100 m*M* Tris-HCl, 100 m*M* sodium chloride, pH 9 5
5 Blocking stock solution. Make up 10% blocking reagent in buffer 1 and heat until almost boiling, mixing occasionally, then autoclave and store at 4°C
6 Wash buffer· Buffer 1 + 0 3% Tween-20. Make fresh, as required
7. RNA extraction buffer 100 m*M* Tris-HCl, 100 m*M* lithium chloride, 10 m*M* EDTA, 1% SDS, pH 8 0
8 MOPS buffer (10X)· 0.4*M* MOPS, pH 7.0, 100 m*M* sodium acetate, 10 m*M* EDTA.
9 SSC (20X)· 0.3*M* sodium citrate, 3*M* sodium chloride, pH 7.0
10 Tris-SDS: 100 m*M* Tris-HCl, 0.2% SDS, pH 8.0
11 Agarose gel loading buffer· 50% glycerol, 1 m*M* EDTA, 0.4% bromophenol blue, and 0 4% xylene cyanol Do not autoclave.

### 2.3. Apparatus

In addition to general laboratory apparatus and a thermocycler for labeling probes by PCR, the following are required.

1 Several steps in the hybridization procedure require the use of heated solutions and constant movement of the solutions over the membrane This can be achieved using a dish on an orbital shaker in an incubator, but is much better done in a purpose-made hybridization oven with rotating hybridization tubes At other stages, when the membrane is incubated in a sealed plastic bag, this should be agitated on an orbital shaker
2 RNA is bound to nylon membranes by heating at 120°C for 15–30 min or by UV-irradiation (*see* **Note 2**) using a purpose-made UV crosslinker In the absence of such a machine, an appropriate UV lamp or transilluminator could be used, but the time of exposure should be determined empirically
3 Glass dishes are required at several stages for incubating membranes These, and all other glassware, should be sterilized before use by dry heat at 145°C

## 3. Method

### 3.1. Labeling Probes by PCR

The use of a Boehringer PCR-DIG labeling kit is recommended. *See* **Note 3** for discussion of some theoretical and practical aspects.

1 Resuspend up to 1 µg template DNA in 5 µL sterile $dH_2O$
2 Mix, on ice, in a PCR reaction tube· 66 5 µL sterile $dH_2O$, 10 µL PCR buffer, 6 µL $MgCl_2$, 10 µL dNTP mix (containing 2 m*M* dGTP, dATP, dCTP, 1.3 m*M* dTTP, and 0.7 m*M* DIG-11-dUTP), 1 µL each of upstream and downstream primers (1 mg/mL).
3 Add to PCR reaction tube 5 µL template DNA and 0 5 µL *Taq* polymerase
4. Mix PCR reaction constituents thoroughly and centrifuge briefly to collect the liquid Overlay with 100 µL of light liquid paraffin, if required by the

thermocycler The following reaction cycles were found to be good using a Perkin-Elmer 9600, but should be altered to suit respective template, primers, and thermocycler

| | |
|---|---|
| Hold at | 95°C for 1 min |
| 5 cycles at | 94°C for 1 min |
| | 55°C for 30 s |
| | 72°C for 30 s |
| 25 cycles at | 94°C for 30 s |
| | 55°C for 30 s |
| | 72°C for 30 s |
| Hold at | 72°C for 5 min |

5 If an aliquot of the reaction mixture is analyzed by agarose gel electrophoresis after amplification, the mol wt of the PCR product will be found to have increased significantly because of incorporation of DIG-11-dUTP, compared to the unlabeled product The concentration of the probe should be determined as described in **Subheading 3.3.** and then stored at 4°C or –20°C (*see* **Note 4**)

## 3.2. Labeling Probes by Random Priming

The use of a Boehringer DIG-High Prime (random priming) kit is recommended. *See* **Note 5** for discussion some theoretical aspects.

1 Resuspend 1 µg template DNA in 16 µL of sterile water
2 Denature the DNA thoroughly by boiling the template for 10 min and chilling on an ice–salt-water mix until cool
3 Add 4 µL of DIG High Prime, mix thoroughly, then centrifuge briefly.
4 Incubate overnight at 37°C
5 Stop the reaction by adding 2 µL 0 2*M* EDTA, pH 8 0
6 Determine the concentration of the probe as described in **Subheading 3.3.** and store at 4°C or –20°C (*see* **Note 4**).

## 3.3. Estimating Concentration of Labeled Probe

1 Make 10-fold serial dilutions of DIG-labeled control DNA in TE buffer from 1 ng/µL to 0 01 pg/µL (equivalent to dilutions of 1 20 1.2,000,000 of the original DNA). Spot 1 µL of each dilution onto a positively charged nylon membrane, approx 60 × 30 mm
2 Prepare a similar dilution series of the labeled probe, direct from the PCR product, and spot 1 µL of each dilution onto the membrane
3 Bind the DNA to the membrane by exposing to UV or by heating (*see* **Note 2**)
4 Equilibrate the membrane for 1 min in wash buffer
5 Incubate membrane at 37°C for 30 min in 50 mL of buffer 2 in a dish
6 Dilute anti–DIG-AP, Fab fragments to 75 mU/mL (1 10,000) in buffer 2 Diluted antibody solutions are stable for 12 h at 4°C A precipitate can form in the antibody solution during storage, this should be removed by brief centrifugation before use Failure to do this can result in background spots on the membrane

7 Incubate the membrane for 30 min in 5 mL of antibody solution at room temperature in a sealed plastic bag
8 Discard the antibody solution and wash the membrane 2 × 15 min with 50 mL of wash buffer in a dish at room temperature
9 Equilibrate the membrane in 10 mL of buffer 3 for 2 min in a dish
10. Dilute chemiluminescent substrate CSPD (25 m*M*) 1 100 in buffer 3 Place the membrane (DNA side up) on one half of a plasic bag, and gently dispense the CSPD substrate onto the surface of the membrane scattering the drops across the surface Approximately 0 5 mL of diluted CSPD is sufficient for a 60 × 30-mm blot Rock the membrane gently to disperse the liquid before lowering a top sheet of plastic over the membrane gently and disperse any bubbles Incubate the filter for 5 min at room temperature without shaking
11 Move the membrane into a fresh plastic bag, allowing excess substrate to drip off during the transfer, seal the bag, and incubate at 37°C for 15 min Do not allow the membrane to dry out during transfer
12 Retain the membrane in the bag and expose to X-ray film for 2–5 min, or as necessary
13. By comparing the intensity of the spots given by the control DNA and the labeled probe, the concentration of the new probe can be estimated (*see* **Note 6**)

### 3.4. Extraction of RNA from Plant Tissues

This method is based on a protocol described in *(2)* **Caution:** It is essential that gloves are worn at all times.

1. Place 0 5 g leaf material (midribs removed) in a 4-mL polyethylene tube (or use less material in a microcentrifuge tube) into liquid nitrogen, and leave until the leaf material is completely frozen before grinding to give a fine powder, using a plastic or glass rod
2 Add 1 mL extraction buffer and 1 mL TE saturated phenol Heat at 80°C for 1 min, mix vigorously for 30 s, incubate at 80°C for 2 min, and mix again
3 Add 1 mL of a 24 1 mixture of chloroform isoamyl alcohol Because chloroform boils at 55°C, it is essential to cool the tubes before adding it Shake vigorously for 30 s.
4 Centrifuge tubes at 16,000*g* for 3 min, remove the upper aqueous phase, and add 1 vol of 4*M* LiCl Mix well and leave overnight at 4°C
5 Centrifuge tubes at 16,000*g* for 3 min and discard aqueous phase Add 200 µL sterile $dH_2O$ to resuspend the pellet, and transfer to a fresh microcentrifuge tube Keep on ice and add 0 1 vol 3*M* sodium acetate and 2 vol of absolute ethanol Mix well and store in the freezer at –20°C overnight, or at –70°C for 30 min.
6 Centrifuge at 16,000*g* for 5 min and discard solution Rinse the pellet in 400 µL 70% ethanol, centrifuge at 16,000*g* for 2 min, and discard solution
7 Remove remaining ethanol from pellets under vacuum, until dry Resuspend and dissolve the pellet in 100 µL Tris-SDS, or more if required Estimate RNA concentration in a spectrophotometer and store at –20°C, after addition of 2 vol of absolute ethanol and 0 1 vol 3*M* sodium acetate, pH 5 8, until ready for use

## 3.5. Preparation of Northern Blots

### 3.5.1. RNA Gels

1 In a 100-mL flask, mix 0 6 g agarose, 5 mL 10X MOPS buffer, and 34 mL sterile distilled water. These constituents should be melted, then cooled slightly before adding 11 mL formaldehyde (37%) Pour the gel and position the sample comb
2 In a 1.5-mL microcentrifuge tube, mix 1 µL 10X MOPS buffer, 3 5 µL formaldehyde (37%), 5 5 µL containing 10 µg of sample RNA in sterile distilled water, and 10 µL deionized formamide Heat samples at 55°C for 15 min and then add 3 µL loading buffer
3 Once the gel is set, place in the gel tank and use 1X MOPS as the electrophoresis buffer
4 Load the samples, including a sample of RNA size marker (*see* **Note 7**), onto the gel and run for approx 2 h at 60 mA, or until the dye is about 3 cm from the bottom of the gel.

### 3.5.2. Transfer of RNA to Nylon Membrane

RNA can be transferred onto a membrane using a vacuum-transfer blotter or by using the following traditional procedure The membrane must not be touched with bare hands.

1 Trim off excess agar from gel, including the loading wells Remove right-hand corner to provide orientation
2 Cut a nylon membrane and two squares of Whatman 3MM chromatography paper to the same size as the gel, and two long strips (cut to the width of the gel) to act as wicks
3 Label, with pencil, the side of the membrane that will be in contact with the gel
4 Place a piece of glass across a large dish containing 300 mL 20X SSC Wet the two longest pieces of filter paper in 20X SSC and drape across the glass and into the SSC in the dish, to act as a wick
5. Carefully place the gel on the wick and smooth out any air bubbles
6 Cover all exposed areas of filter paper with cling film
7. Wet the membrane in 10X SSC (*see* **Note 8**) and place on top of the gel, smoothing out any air bubbles and making sure that the corners are well-matched
8. Wet the two squares of filter paper in 20X SSC, place on top of the membrane, and smooth down gently
9 Place a 70-mm stack of absorbent paper towels on top, weigh down evenly with 500 g weights, and leave to transfer overnight
10 Bind the RNA to the membrane by exposing to UV or by heating (*see* **Note 2**) The membrane should be irradiated on both sides before hybridization with DIG probes, as described in **Subheading 3.7.**

## 3.6. Preparation of RNA Dot Blots

1. Prepare serial dilutions of total RNA extracts from leaves in sterile $dH_2O$ (typically from 5 µg/µL to 0 5 pg/µL). 2µL of each dilution should be spotted onto a piece of positively charged nylon membrane

2. Bind the RNA to the membrane by exposure to UV or by heating (*see* **Note 2**) The membrane should be irradiated on both sides before hybridization with DIG probes, as described in **Subheading 3.7.** Following nucleic acid detection, quantitative estimates of the amount of RNA can be made (*see* **Note 9**)

### 3.7. Hybridization of DIG-Labeled DNA Probes to RNA on Nylon Membranes

*See* **Note 10** for discussion of hybridization buffers

1 Prewarm to 37°C the roller containing 20 mL DIG Easy Hyb solution and incubate the membrane for 30 min to prehybridize (*see* **Note 11**)
2 Denature the DIG-labeled DNA probe by boiling for 10 min and then rapidly chilling on an ice–water mix Add probe to fresh prewarmed DIG Easy Hyb solution (2.5 mL is sufficient for a 100-$cm^2$ membrane, if using a roller, the minimum volume required is 8 mL) and mix gently. Do not allow foaming to occur, because bubbles cause increased background
3 Pour off and discard the prehybidization solution from the membrane and place it in a plastic bag; immediately add the probe in DIG Easy Hyb Do not allow the membrane to dry out
4. Incubate overnight at 37°C
5 Pour off the hybridization probe solution and retain Probes can be re-used three to four times after storage at –20°C When reusing a probe diluted in DIG Easy Hyb, it must be denatured at 68°C for 10 min and fast chilled before use (*see* **Note 12**) Hybridized nucleic acid should be detected as described in **Subheading 3.8.**

### 3.8. Detection of Hybridized Nucleic Acid

1 After hybridization, wash the membrane for 2 × 5 min at room temperature with at least 50 mL 2X SSC containing 0 1% SDS to remove unbound probe It is preferable to do this in a dish on an orbital shaker, rather than using a hybridization oven. Ensure there is enough buffer for the membrane to move freely
2 Wash 2 × 15 min at 68°C with at least 50 mL of 0.1X SSC containing 0 1% SDS The buffer should always be prewarmed, and, if using a hybridization oven, both the hybridization tube and buffer should be preheated (*see* **Note 13**)
3 Now proceed from **steps 4–11** of **Subheading 3.3.** Use 1 mL of diluted CSPD substrate for a 100-$cm^2$ blot.
4. Expose to standard X-ray film for 30 min–24 h, as necessary. Exposures can be obtained for up to 2 d after the addition of the chemiluminescent substrate

## 4. Notes

1 Filtration of many solutions is recommended by Boehringer, but we commonly autoclave without apparent problems
2 RNA and DNA can be bound to the membrane by heating at 120°C for 30 min or by exposure to 120,000 μJ/$cm^2$ UV. Always UV crosslink both sides of the membrane, because this will reduce background

3 PCR can be used to prepare DIG-labeled DNA probes DNA probes are efficiently labeled with DIG-11-dUTP using PCR, because small amounts of DNA (100 ng–1 μg) can be directly amplified and labeled in a single reaction. Although the PCR could be done directly on intact plasmid DNA, Boehringer have recommended that insert be restriction-digested from vector sequences, followed by phenol chloroform extraction and ethanol precipitation before labeling We have found this improves the probe sensitivity, compared to using undigested plasmid

The optimal PCR conditions have to be adapted and optimized to each template–primer combination The concentrations of $MgCl_2$, enzyme, template, and primers, and the thermocycler reaction times we have given may not suit all situations It is possible and convenient to use reduced PCR volumes (typically 50 μL) when determining optimal conditions for making a new probe

4 Probes in regular use can be stored at 4°C, or at –20°C, if intended for long-term storage

5 Random priming is a fast and efficient method for preparation of probes and is well-suited for labeling relatively long probes However, there may be only limited introduction of labeled nucleotide into the probe Using a Boehringer DIG-High Prime kit, DIG-labeled probes can be generated within an hour, but, for increased yield, an overnight reaction is preferable It is important to digest the vector to remove the target insert prior to labeling; labeled vector sequences can lead to nonspecific hybridization

6 Typically, we use a DNA probe concentration of 5–25 ng/mL to detect RNA However, the optimum concentration of a new probe should be determined, taking background signal into account

7 The samples should include a lane containing a conventional RNA marker, which can be cut off before transfer, and ethidium bromide stained An alternative, and preferable, method is to use DIG-labeled RNA mol-wt marker supplied by Boehringer; there are three size ranges available This has the advantage that it will be transferred during blotting and will be visible on the X-ray film alongside the sample bands DIG-labeled mol-wt marker should be heated at 65°C for 10 min before being placed on ice, other details for using mol-wt markers can be found in the manufacturer's technical leaflet

8. It is not necessary to prewet nylon membrane, but, if required, it wets faster in 10X SSC than in 20X SSC

9 Estimates of transcript RNA concentration can be made by comparing the intensities of hybridized nucleic acid spots with those given by dilutions of a known concentration of purified RNA Estimates of relative amounts of RNA can also be determined by comparing the intensities given by different samples. The range of dilutions that is suitable for previously untested RNA extracts, should be determined empirically It is very important to include RNA samples from nontransgenic plants to act as controls for nonspecific hybridization

10 There are various buffers that can be used for hybridization Dig Easy Hyb is nontoxic and does not contain formamide. It is ready to use and is both DNase- and RNase-free The optimum hybridization temperature should be estimated

empirically and depends on whether the target is DNA or RNA, the GC content, and homology of probe to target, further details can be found in **ref. *1*** Although the use of DIG Easy Hyb buffer is probably the easiest option, if buffer containing formamide is required, the following can be used: 5X SSC, 0 1% lauryl-sarcosine, 0 02% SDS, 1% blocking reagent, and 50% deionized formamide

11 Prehybridization can be longer than 30 min, but do not exceed 2 h

12. Probe denaturation is more efficient if done in a water bath rather than a dry block.

13 Unbound probe is removed in washing **steps 1** and **2** If using probes >100 bp, the washes in **step 2** must be at 68°C; for shorter probes, the washing temperature must be determined empirically. Membranes are most easily washed by shaking in a dish in an incubator, rather than in a hybridization oven. An exception to this are the washes in **step 2** at 68°C, which is difficult to achieve in an incubator It is important to preheat the roller and buffer before use in this step. Never allow the membrane to dry out at any time, because this will increase background

## References

1. DIG System User's Guide for Filter Hybridization Boehringer Mannheim, Mannheim, Germany
2 Verwoerd, T C , Dekker, D M M , and Hoekema, A (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res* **17,** 2362

# 46

# Assaying Levels of Plant Virus by ELISA

**Roy Copeland**

## 1. Introduction

Enzyme-linked immunosorbent assay (ELISA), since its use with plants was described by Clark and Adams *(1)*, has become the most popular method for detection of viruses in plants, because of its simplicity and wide applicability. Used mainly to confirm presence or absence of infection, it can be adapted to estimate concentration of an antigen, e.g., viral capsid protein, in plant sap. The procedure described in detail here is that for double antibody sandwich (DAS) ELISA with alkaline phosphatase-conjugated antibody, which suffices for most occasions. However, the principles outlined will apply equally well to indirect ELISA, using an antispecies conjugate, or to plate trapped antigen ELISA, should those variants be the method of choice for a specific viral protein.

The text is worded for those who already have, or can purchase, viral-specific IgG and enzyme-conjugated antibodies. Preparation of antibodies is described in Chapter 29 of this volume. Optimization of IgG and conjugated antibody dilutions is fully described by Hill *(2)* and Converse and Martin *(3)*.

## 2. Materials

1. 96-Well flat-bottomed polystyrene or polyvinyl microtiter plates suitable for binding of IgG
2. Viral protein-specific IgG for coating wells.
3. Viral protein-specific antibody conjugated to alkaline phosphatase
4. Coating buffer: 0.1 *M* carbonate buffer, adjusted to pH 9.6 (1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$ per L).
5. Sample buffer: Normally phosphate-buffered saline (PBS) pH 7.4: 8 g NaCl, 0.2 g $KH_2PO_4$, 2.9 g $Na_2HPO_4$ 12 $H_2O$ per L, with the addition of 0.05% Tween-20 and 2% polyvinylpyrrolidone, mol wt 40,000.

From *Methods in Molecular Biology, Vol 81: Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by: G. D. Foster and S. C. Taylor © Humana Press Inc., Totowa, NJ

6 Conjugate buffer Sample buffer with the addition of 2 g/L ovalbumin (Sigma, Grade V)
7 Substrate buffer 1*M* diethanolamine adjusted to pH 9 8 with 2*N* HCl Buffers should be kept refrigerated An antimicrobial agent, e g , 0 2 g/L of $NaN_3$, should be added to coating, sample, and conjugate buffers if prolonged storage is anticipated (**Caution:** $NaN_3$ is highly toxic )
8 Wash buffer PBS containing 0 05% Tween-20 (PBS-Tween) Wash buffer can be stored as a 10X concentrate without preservative and used freshly diluted
9 Substrate *p*-nitrophenyl phosphate (1 mg/mL) is the substrate normally used with alkaline phosphatase Powder or tablets work equally well
10 Purified viral protein or virions, concentrated at least to 0 1 μg/mL

## 3. Method

Since the aim is to compare concentrations of viral protein in many samples of tissues, perhaps assayed over substantial periods of time, standardization and faithful replication of procedures at all stages from growth of plants to the completion of ELISA are required to maximize precision in resultant data.

### 3.1. Collection of Leaf Tissue and Extraction of Sap

1 Either a disk of tissue from the lamina of a leaf or an entire leaflet or leaf can be the standard unit of tissue for assay If sampling spans days or weeks, tissue should be collected at the same time each day from plants maintained under uniform conditions of photo period, temperature, nutrition, and hydration

It is advisable to refrigerate sampled tissue immediately after collection if the interval before sap extraction will exceed a few minutes

2 Sap may be extracted mechanically (in which case, it is necessary to know the dry matter content of the tissue) and aliquots added to sample buffer predispensed into microtiter wells, or tissue can be macerated in a proportionate volume of sample buffer before transfer of aliquots to microtiter wells Either way, the interval from sap extraction to placement in wells should be minimized

### 3.2. ELISA Procedure

1 Add trapping antibody diluted appropriately in coating buffer Normally, a 1 μg/mL IgG concentration is used Any volume between 50 and 200 μL per well can be chosen It should, though, be equal to or slightly greater than the volumes used in subsequent stages of the procedure Incubate at 4°C overnight
2 Mechanically or manually empty trapping antibody solution from the wells and wash three times with PBS-Tween (*see* **Note 1**) Washed plates should be inverted and tapped onto tissue paper to dislodge droplets remaining in wells Plates can be used immediately for the next stage, or, alternatively, wrapped in polythene to prevent drying, and stored for days at 4°C or for up to 6 mo at –20°C
3 If necessary, at this stage and/or after washing out of test antigen, plates can be treated to block any remaining nonspecific reactive sites on the polystyrene surfaces as a means of reducing high background-absorbance values (*see* **Note 2**)

4 Add test antigen diluted in sample buffer Any convenient volume between 50 and 200 µL is suitable, but it should not be greater than that used in coating the plate For assay of virus capsid protein in infected sap, the optimum dilution of sap may range from 1.5 to 1:1000 and has to be determined by a preliminary assay (*see* **Notes 3** and **8**) The layout of test samples and calibration samples on a plate is discussed in **Note 4** Preferably, incubate dilute sap overnight at 4°C Alternative incubation periods are 3–4 h at 25°C or 2–3 h at 37°C
5 Wash thoroughly with PBS-Tween to remove all traces of nonspecifically bound antigen Wells should be filled and emptied at least three times
6 Add alkaline phosphatase-conjugated antigen-specific antibody diluted in conjugate buffer warmed to the intended incubation temperature Use at a dilution previously determined to give maximum discrimination between positive and negative samples Volume per well is that used for antigen samples. Incubate for 3–4 h at 25–37°C, or overnight at 4°C (*see* **Notes 6** and **7**)
7 Wash thoroughly with PBS-Tween.
8 Add freshly prepared *p*-nitrophenyl phosphate substrate, 1 mg/mL (preferably the same volume/well as in **step 6**), diluted in substrate buffer warmed to room temperature Incubate at room temperature, away from direct sunlight, and record absorbance at 405 nm, when color has developed, usually after 15–60 min (*see* **Note 5**) If necessary, development of color can be halted by the addition of 50 µL 3*N* NaOH/well.

### 3.3. Calculation of Virus Protein Concentration in Test Samples

1 Prepare a calibration curve for purified virus protein based on mean absorbance values obtained for each concentration of purified viral protein
2. Calculate mean absorbance values for each virus-inoculated sample and subtract the absorbance value obtained for the noninoculated (virus-free) control
3 Using the calibration graph, convert absorbance values into ng virus protein per well
4 Allowing for dilution of sap and volume per well, calculate weight of viral protein per unit weight of plant tissue or unit volume of sap For example, in **Fig. 1A**, the 1:1000 dilution of sap yielded an absorbance value of 0 6, which **Fig. 1B** shows is equivalent to 62 ng viral protein Because each well contained 50 µL of diluted sap, the calculation for concentration of virus protein is 62 ng × 1000 (dilution factor) × 1000 µL/50 µL = ng virions/mL of undiluted sap = 1 24 mg PVX virions/mL of undiluted sap.

A portion of the leaves from which sap was extracted was weighed, dried, reweighed, and calculated to have 14.4% dry matter The concentration of virions therefore can be recalculated as· 1 24 × (100 – 14 4)/100 = 1 06 mg PVX virions/g fresh wt of leaf

## 4. Notes

1. At all stages of ELISA, thorough washing of wells is essential to minimize background color. Flaws in the performance of mechanical/automatic washers are a

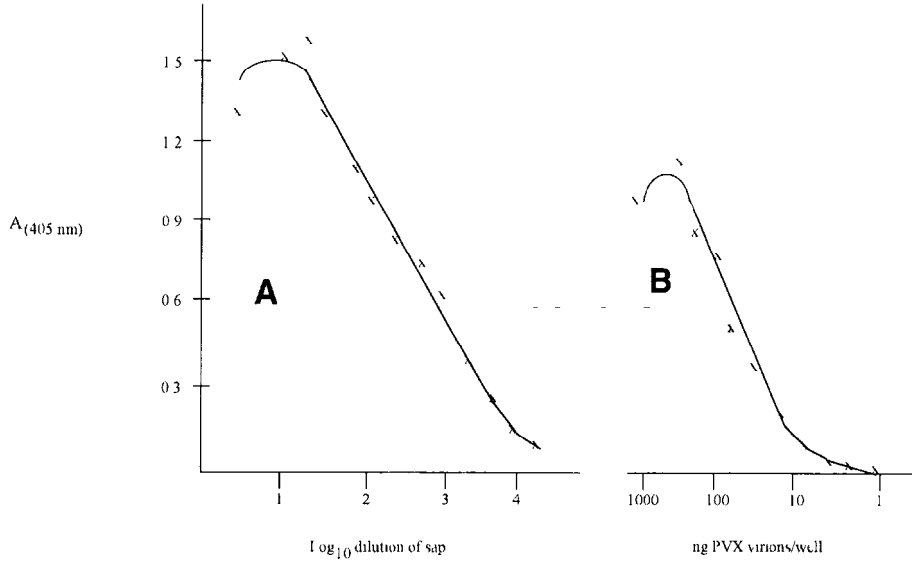



Fig 1 Relationship between concentration of viral protein and absorbance in DAS-ELISA **(A)** Diluted sap of PVX-infected leaves **(B)** Purified PVX virions diluted in sample buffer

common cause of elevated absorbance values in rows, columns, or single wells of microplates Turning a microtiter plate through 180° and repeating the wash program is a simple way of ensuring that all wells are washed thoroughly when using a mechanized washer. Plant debris that settles on the floor of wells and is not dislodged during washing increases background color Mechanical agitation of plates at the end of **step 4** of the ELISA procedure should overcome the problem Even if using a plate-washing machine that will aspirate contents of wells, it is advisable to manually empty each plate after incubation of diluted sap and antibody-enzyme conjugate, and wipe dry the top surface, before washing with PBS-Tween Errors caused by uneven background color development are more easily detected if there are at least duplicate wells of each sample on a microtiter plate

2 Agents commonly reported to be effective in blocking uncoated reactive sites on the surfaces of wells are· 0 2–2% solution of powered nonfat milk, bovine serum albumin, ovalbumin, or gelatine diluted in PBS-Tween and incubated for 1–2 h at 25°C; 1 μg/mL polyvinyl alcohol (Sigma P8136) in PBS-Tween, incubated for 1 min at room temperature

3 A plot of absorbance against $Log_{10}$ antigen concentration in DAS ELISA will produce a sigmoid curve with depressed absorbance values at the very highest concentrations of antigen (**Fig. 1**) Accurate quantitative analysis is possible only in the median range of antigen concentration, where absorbance responds lin-

early to change in antigen concentration. Thus, when assaying concentration of viral protein in transgenic plants, a preliminary assay has to be run with serial dilutions of sap from virus inoculated untransformed plants of the parent genotype inoculated and grown in identical conditions to determine the range of dilutions that lie within the linear phase of the sigmoid curve A similar assay is needed to quantify the response to concentrations of purified virions or viral protein (**Fig. 1B**) In the linear phase, both curves should have equal slopes

The dilution of sap chosen for assay of transgenic plants should produce absorbance values that fall within the linear phase of the associated calibration assay of purified viral protein If it is anticipated that transgenic plants will have a lower virus content than the parent genotype, then a dilution at the upper end of the linear phase would be appropriate (e g , 1·100 in the assay depicted in **Fig. 1A**). Two or more dilutions of sap of test samples may have to be included if the plant material to be assayed is expected to have a wide variation in viral concentration

4. The distribution of samples on a microtiter plate is a matter of individual choice, often constrained to a degree by the software program in the colorimeter/plate reader used to measure absorbance. Each plate, though, should have wells to which only reagents have been added, and against which background absorbance the plate is blanked in a plate reader, wells with a range of concentrations of purified viral protein diluted in sample buffer; wells with sap from untransformed, virus-inoculated plants; wells with sap from untransformed, virus-free plants, wells with sap from virus-inoculated plants of each transgenic line, and wells with sap from virus-free plants of each transgenic line Bunch et al *(4)* have described in detail how allocation of wells on a 96-well microtiter plate between calibration and test samples influences accuracy and efficiency in quantitative ELISA Their calculations are complex and need recalculation for every protocol, but, simplified, they show that (1) the calibration assay should contain at least four concentrations of viral protein, distributed over the linear phase of the absorbance curve, and that (2) all test samples should be included on each plate It is more efficient to split replicates between plates rather than test samples
5. Prior to reading color development, wipe the top surface of each plate to prevent substrate solution from contaminating the photocells in the reader.
6. Evaporation from wells during incubation of plates can be minimized by using proper plate covers or by simply placing an empty used plate on top and putting the stack of plates in a polythene bag.
7. If an indirect ELISA using an antispecies antibody–enzyme conjugate is required, then the second virus protein-specific antibody is incubated at **step 6** of the ELISA procedure, and, after washing, the antispecies conjugate is incubated in wells for 3–4 h at 25°C or 2–3 h at 37°C
8. If plate-trapped antigen ELISA is necessary, determination of the optimum dilution of sap in buffer is still required as a prelude to the actual assay for viral protein

## References

1 Clark, M. F. and Adams, A N (1977) Characterıstics of the mıcroplate method of enzyme-lınked ımmunosorbent assay for the detectıon of plant vıruses *J Gen Vırol* **34,** 475–483
2 Hıll, S A (1984) *Methods in Plant Virology* Blackwell, Oxford, UK
3 Converse, R. H and Martın, R R (1990) ELISA methods for plant vıruses, ın *Serologıcal Methods for Detectıon and Identıfıcatıon of Viral and Bacterıal Plant Pathogens* (Hampton, R , Ball, E , and De Boer, S , eds ), APS, MN, pp 179–196
4 Bunch, S D., Rocke, D M , and Harrison, R. O (1990) Statıstıcal desıgn of ELISA protocols. *J Immunol Methods* **132,** 247–254

# 47

# Detection of Plant RNA Viruses by Nonisotopic Dot-Blot Hybridization

**Vicente Pallás, Paloma Más, and Jesús A. Sánchez-Navarro**

## 1. Introduction

Unlike for fungal and bacterial diseases, no direct method for the control of viral diseases is yet available. The early detection of plant viruses constitutes, therefore, one of the main ways of controlling these diseases, and so sensitive detection systems are essential. Until now, methods based on the protein component of the viral particle have been mainly used in plant virus detection *(1,2)*. The application of recombinant DNA technology to plant virology has permitted the use of diagnostic methods based on the genomic component of viruses. Nucleic acid hybridization has proved to be a very reliable and sensitive technique in plant virus diagnosis (for a review, *see* **refs.** *3* and *4*). The most common method for molecular hybridization is the dot-blot hybridization technique, which involves the direct application of a nucleic acid solution to a solid support, such as nitrocellulose or nylon membranes, and detection with appropriate probes. Until very recently, these probes usually consisted of nucleic acids labeled with radioactive precursors, but, because of the risks and safety precautions required, the use of radioactively labeled probes has been restricted to specialized laboratories.

The use of nonradioactive precursors to label nucleic acids has made the molecular hybridization technique more accessible and is being used in more virus–host combinations. Among these nonradioactive precursors, those derived from biotin and digoxigenin molecules are the most widely used. The biotinyl labeled nucleic acids are recognized with great efficiency by avidin or its microbial analog, streptavidin, taking advantage of the exceptionally high affinity of the avidin–biotin complex The main disadvantage of this system is that, when sap extracts are used, the endogenous biotin can cause false posi-

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

tives, or, alternatively, the presence of glycoproteins that bind avidin- or biotin-binding proteins can give unworkably high background. Another widely used molecule to nonradioactively label nucleic acids is the hapten digoxigenin, which is bound via a spacer arm (11 carbon residues) to uridin-nucleotides and is incorporated enzymatically into nucleic acids by standard methods. The protocol described here is based on probes labeled with digoxigenin. The fact that methods based on the biotin–avidin complex have not been included in this chapter does not mean that they are less efficient, but only that the authors are less familiar with them. The reader is referred to excellent reviews and original papers for details on the biotin–avidin system *(5–9)*.

The following procedure is divided into three steps The first involves the synthesis of the labeled probe by incorporating the digoxigenin into a cRNA by means of an in vitro transcription reaction, or into DNA by the random-priming method The second part describes the preparation of the samples and the molecular hybridization reaction with the appropriate probe. After hybridization and blocking steps, bound probes are detected by high-affinity antibody Fab-fragments coupled to alkaline phosphatase. The third part implies the development of the detection method. Although chemiluminescent detection methods have been reported to be much more sensitive than colorimetric methods, the latter are also described, because of their convenience for testing field samples in less well equipped laboratories.

## 2. Materials

### 2.1. cRNA Probes

1 10X Transcription buffer: 400 m*M* Tris-HCl, pH 8 0 (20°C), 60 m*M* $MgCl_2$, 100 m*M* dithiothreitol (DTT), 20 m*M* spermidine, 100 m*M* NaCl (Boehringer Mannheim, Mannheim, Germany). Store at –20°C
2 10X Digoxigenin-RNA (DIG-RNA) labeling mixture 10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 6 5 m*M* UTP, 3 5 m*M* Dig-UTP, pH 7 5 (20°C) (Boehringer Mannheim) Store at –20°C.
3 RNase inhibitor (40 U/µL) (Promega, Biotec, Madison, WI) Store at –20°C
4 RNA polymerase T7, T3, or SP6 (Boehringer Mannheim) (40 U/µL) Store at –20°C
5. DNase I, RNase-free (20 U/µL) (Promega) Store at –20°C
6. 0.2*M* EDTA, pH 8 0 Store at room temperature
7 TE buffer 10 m*M* Tris-HCl, pH 8 0, 1 m*M* EDTA. Store at room temperature
8. 4*M* LiCl. Store at 4°C.

### 2.2. DNA Probes

1 10X Hexanucleotide mixture 0 5*M* Tris-HCl, pH 7.2 (20°C), 0.1*M* $MgCl_2$, 1 m*M* dithioerythritol (DTE), 2 mg/mL bovine serum albumin (BSA), hexanucleotides 62 5 $A_{260}$ U/mL, pH 7 2 (20°C) (Boehringer Mannheim). Store at –20°C.

2 10X DIG-DNA labeling mixture. 1 m*M* dATP, 1 m*M* dCTP, 0 65 m*M* dTTP, 0 35 m*M* Dig-dUTP, pH 7 5 (20°C) (Boehringer Mannheim) Store at –20°C
3. Klenow enzyme (2 U/μL) (Boehringer Mannheim) Store at –20°C
4 4*M* LiCl Store at 4°C

### 2.3. Dot-Blot Hybridization

1 Mortars.
2 50 m*M* Sodium citrate, pH 8.5. Autoclaved Store at room temperature
3 Nylon membrane, positively charged (Boehringer Mannheim)
4 20X SSC. 3*M* NaCl, 0 3*M* sodium citrate, pH 7.0. Autoclaved Store at room temperature
5 Blocking stock solution 10% (w/v) blocking reagent (Boehringer Mannheim) in buffer 1 (*see* **Subheading 2.4., item 1**); autoclaved and stored at 4°C
6 Prehybridization solution 50% (v/v) deionized formamide, 5X SSC, 2% (w/v) blocking reagent (added from 10% blocking stock solution), 0 1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS) Store at –20°C
7 Washing solution I 2X SSC, 0 1% (w/v) SDS.
8 Washing solution II· 0 1% SSC, 0 1% (w/v) SDS

### 2.4. Immunological Detection

1 Buffer 1. 0.1*M* maleic acid, 0 15*M* NaCl, pH 7 5 (20°C), adjusted with concentrated NaOH, autoclaved
2 Washing buffer 0.3% Tween-20 (Sigma, St Louis, MO) in buffer 1
3 Buffer 2· 1% blocking reagent (added from 10% sterile stock solution) in buffer 1
4. Buffer 3. 0.1*M* Tris-HCl, pH 9 5, 0 1*M* NaCl, 50 m*M* $MgCl_2$ Discard the solution if a precipitate appears after a long storage period
5. Antibody-conjugated solution freshly prepared Dilute 1.10,000 the sheep antidigoxigenin Fab-fragments conjugated to alkaline phosphatase (750 U/mL) (Boehringer Mannheim), in buffer 2. Diluted solution is stable only for 12 h at 4°C Undiluted preparation are stable at 4°C Do not freeze
6 75 mg/mL Nitroblue tetrazolium (NBT), in 70% (v/v) dimethylformamide, and 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP), in dimethylformamide (Boehringer Mannheim) Store at –20°C
7 CSPD® substrate (10 mg/mL) (Tropix, Bedford, MA) Store at 4°C and protected from light
8 X-Ray films

## 3. Method

### 3.1. Synthesis of the Probe

The two methods described here for preparing the nonisotopic label require that part of the viral sequence is cloned into the polylinker site of a plasmid vector that contains a promoter for SP6, T7, or T3 RNA polymerase. For RNA probes, the plasmid vector must be digested with an appropriate restriction

endonuclease that leaves the insert downstream of the RNA polymerase promoter If possible, select a restriction enzyme that generates a 5' overhang end, otherwise, longer-than-unit transcripts may be obtained. Following digestion, the linearized plasmid is extracted with phenol·chloroform before using the DNA for transcription reactions. For DNA probes, the complementary strand is synthesized by using a mixture of random hexanucleotides to prime DNA template. Although the linearization of the plasmid is not necessary, it is recommended because it increases the efficiency of the labeling When no previous information about the viral primary structure is available, a digoxigenin-labeled cDNA probe can be synthesized from viral RNA by priming with random hexamers and using reverse transcriptase by standard protocols ***(10)***.

### 3 1.1 cRNA Probes

1 To a microcentrifuge tube containing 1 μg of linearized DNA template, add the following. 2 μL of 10X transcription buffer, 2 μL of 10X DIG-RNA labeling mixture, RNase inhibitor (40 U), SP6, T7, or T3 RNA polymerase (40 U), and make up to a final volume of 20 μL with sterile water All the components must be added at room temperature, since DNA can precipitate at 4°C in the presence of spermidine
2 Centrifuge briefly and incubate for 2 h at 37°C
3 (Optional) Add DNase I, RNase-free (20 U), and incubate for 15 min at 37°C This treatment is not usually necessary, because the DIG-labeled RNA transcript is 10-fold in excess of the template DNA
4 Add 2 μL 0.2*M* EDTA, pH 8.0, to stop the reaction
5 Precipitate the labeled RNA with 2.5 μL 4*M* LiCl and 2 5 vol of ethanol
6 Centrifuge at 12,000*g* and resuspend the RNA sample in TE buffer or sterile water (*see* **Notes 1** and **2**).

### 3.1.2. DNA Probes

1 Denature the DNA by heating it at 95°C for 10 min Rapidly submerge the tube in an ice bath
2. Add to a microcentrifuge tube the following reagents 10 ng–3 μg freshly denatured DNA, 2 μL of 10X hexanucleotide mixture, 2 μL of 10X DIG-DNA labeling mixture, Klenow enzyme (2 U), and make up to a final volume of 20 μL with sterile water.
3 Mix gently, centrifuge briefly, and incubate for at least 60 min at 37°C
4 Add 2 μL of 0.2*M* EDTA, pH 8 0, to stop the reaction.
5 Precipitate, and recover the labeled DNA, as described above

## 3.2. Preparation of the Samples and Hybridization

1 Homogenize the leaf tissue in a prechilled mortar with 2 vol 50 m*M* sodium citrate, pH 8.5 Alternatively, the tissue can be homogenized in resistant plastic bags (*see* **Note 7**)

2 The extracts are clarified by centrifugation in a microcentrifuge at 15,900*g* for 15 min The supernatant is then ready for application to a nylon membrane Denaturation of the samples at this step can help to increase sensitivity (*see* **Note 3**).
3 Cut a piece of a nylon membrane to the appropriate size Before removing the two pieces of paper that protect the membrane, mark it into 1 × 1-cm squares with a pencil The membrane below will be sufficiently marked by the pencil's pressure for the squares to be visible. Cut a corner of the membrane so that the samples can be located after detection. It is important to handle the membrane with gloved hands or with forceps to avoid undesirable background
4. A 5-µL sample of the supernatant obtained in **step 2** is then applied to each marked square of the membrane Nylon membranes does not need any previous treatment. Nitrocellulose membranes must be prepared by presoaking in water and then in 20X SSC. They must also be dried before being loaded with RNA Larger volumes can be applied under vacuum with the aid of a Bio-dot blotting apparatus (Bio-Rad, Hercules, CA). The sample volume must be then adjusted to avoid saturation of the membrane The nucleic acids are bound covalently to the membrane by baking at 80°C for 2 h (*see* **Note 5**)
5 Nonspecific binding sites are blocked by incubating the membrane in the prehybridization solution (10 mL/100 $cm^2$ of membrane) twice for 1 h at 68°C
6 The prehybridization mixture is then removed and replaced by 10 mL/100 $cm^2$ of the same solution, plus 0 2–0 5 µg/mL of the DIG-labeled RNA probe, and incubated overnight at 68°C DNA–DNA hybridizations must be made at 42°C, hybridizations of the RNA probes to DNA blots are usually performed at 50°C Preferably, the prehybridization and hybridization steps are carried out in a hybridization oven, if available Alternatively, they can be carried out in heat-sealable plastic bags in a water bath, with gentle shaking, in which case care must be taken that no air bubbles are trapped in the plastic bag The solution containing the probe can be reused several times without loss of sensitivity In addition, probes stored at –20°C in the hybridization solution can be used for several months without significant loss of sensitivity In our case, probes stored up to 2 yr, which have had no more than four or five uses, gave satisfactory results
7 Finally, the membrane is washed 2 × 5 min at room temperature with washing solution I, followed by 2 × 15 min at 68°C in washing solution II, to eliminate the nonhybridized probe

### 3.3. Immunological Detection

All the steps are performed at room temperature with gentle shaking. Put the membrane in a plastic box or in hybridization bags, and make sure that the solution covers the membrane totally.

1 The membrane is washed briefly (approx 2 min) in washing buffer
2 Blocking reagent (buffer 2) is then added and the membrane is incubated for 30 min at room temperature to block nonspecific detection

3 The membrane is then incubated at room temperature for 30 min with antibody-conjugated solution
4 To eliminate the excess of unbound antibody, the membrane is incubated in washing buffer 2 × 15 min and then equilibrated for 3 min with buffer 3 Optical or chemiluminescent detection methods can be developed, depending on the type of substrate used

### 3.3.1 Colorimetric Detection

1 The membrane is incubated in a plastic bag in the dark, without shaking, with a freshly prepared solution containing NBT (0 3375 mg/mL) and BCIP (0 175 mg/mL), in buffer 3 in a final volume of approx 5 mL/100 $cm^2$ of membrane.
2 Remove the substrate solution when a purple color appears (15 min–12 h) The color reaction is stopped by washing the membrane in TE buffer The results can be documented by photocopying or by photography

### 3 3.2. Chemiluminescent Detection

1 The chemiluminescent substrate CSPD® (10 mg/mL) is diluted 1 100 in buffer 3 The membrane is incubated for 5 min with 5 mL of the above freshly prepared solution/100 $cm^2$ in a plastic bag in the dark
2 Cut the plastic bag at the top and remove the substrate solution (it can be re-used several times) Using forceps, slide the membrane up the sides of the bag so that any excess liquid remains in the bag (do not allow the membrane to dry) The membrane is then sealed damp in a new plastic bag and incubated for 15 min at 37°C Make a first exposure to X-ray film at room temperature for approx 15 min Additional exposures may be taken, since luminescence continues for at least 24 h Do not let the membrane dry if reprobing is intended (*see* **Note 6**) Store the membranes at 4°C in 2X SSC

## 4. Notes

1 An easy way to double-check that the digoxigenin has been incorporated into the transcript RNA is to compare the electrophoretic mobility in TBE-agarose gels of the transcription products obtained in the presence and absence of the precursor DIG-UTP If the digoxigenin has been incorporated into the cRNA, its electrophoretic mobility will be slower than that of the unlabeled transcript Alternatively, transcription products can be serially diluted and spotted on nylon membranes, which can be developed as described in the general method
2 In some cases, no or very low yields of digoxigenin-labeled transcripts are obtained A dose-dependent inhibition of SP6, T7, and T3 RNA polymerases by the DIG-UTP precursor has been previously described ***(11)***. In addition, some templates are not suitable for the transcription reaction In this situation, the manufacturer of DIG-UTP recommends recloning the cDNA, so that another RNA polymerase can be used However, Heer et al ***(11)*** have shown that lowering the relative amount of DIG-UTP could circumvent this problem In our hands, templates having a size in the range of 1–2 7 kb gave the best yields when using

a ratio of 0.28.0.72 (DIG-UTP UTP) vs the one recommended by the manufacturer (0 35:0 65) However, since no direct correlation between a specific sequence pattern and transcription efficiency has been observed, the optimal DIG-UTP:UTP ratio must be determined experimentally for a given template, if synthesis of the probe continues to be a problem

3 If sensitivity is poor when using clarified sap extracts to detect RNA viruses, samples can be denatured by heating at 60°C for 15 min in the presence of formaldehyde, since this treatment helps to increase the hybridization signal slightly ***(12)***. Additionally, samples can be phenol-extracted and concentrated by ethanol precipitation. However, this last step is not recommended for routine diagnosis, because the procedure is obviously more laborious In addition, solutions containing phenol are highly toxic and special safety precautions must be taken

4. When possible, chemiluminescent detection should be the method of choice because of its higher sensitivity over the colorimetric one In addition, when clarified sap extracts are used, the remainder of the natural green-brownish color of leaves on the membranes interferes directly with the colorimetric detection, probably because of the reduction of the nitroblue tetrazolium by components of the plant sap, the light emission is not altered by the presence of these components.

5 If nylon membranes are used, the sensitivity of the chemiluminescent detection method can be increased by UV crosslinking of RNA-containing plant samples, which results in a 5- to 10-fold increase over the standard baking methods When no UV source is available, the samples can be fixed to the membrane by baking for 15–30 min at 120°C or for 2 h at 80°C

6. In some applications (for instance, to diagnose several viruses on the same membrane), it would be desirable to have the possibility of reprobing Unfortunately, digoxigenin-labeled cRNA probes tend to remain bound to RNA dot blots following stripping treatments that remove $^{32}$P-labeled cRNA probes, thus making reprobing not recommended when using this kind of probes. Then, it is advisable to duplicate membranes with equivalent samples and probe them with the different probes

7 To make the nonisotopic dot-blot hybridization technique more accessible to nonspecialized laboratories, an alternative procedure can be applied to prepare and clarify field samples ***(13)***. Sap extracts are obtained by homogenizing the tissue with 1 vol 50 m*M* sodium citrate in a resistant plastic bag with a hand model homogenizer Using sap-impregnated cotton buds, the samples are then applied to nylon membranes by uniformly pressing them until a lateral diffusion is observed. The nucleic acids are then bound to the membrane and hybridized, as previously described This procedure avoids the need for centrifugation to clarify the samples and the use of micropipets to load them. Although the material directly applied on the central area is not accessible to the luminescent substrate, the diffused material gives a very specific and reliable doughnut-like hybridization signal, which is just as sensitive as clarifying the samples by centrifugation.

## References


1 Clark, M F., Lister, R M , and Bar-Joseph, M. (1986) ELISA techniques *Methods Enzymol* **118,** 742–767

2 Van Regenmortel, M H V and Dubs, M C (1993) Serological procedures, in *Diagnosis of Plant Virus Diseases* (Matthews, R. E. F , ed.), CRC, Boca Raton, FL, pp. 159–214

3 Hull, R (1993) Nucleic acid hybridization procedures, in *Diagnosis of Plant Virus Diseases* (Matthews, R E F., ed ), CRC, Boca Raton, FL, pp 253–271

4 Matthews, R E F (1991) *Plant Virology*, 3rd ed Academic, San Diego, CA

5. Gillman, I C (1987) Non-radioactive probes for specific DNA sequences *Trends Biotech* **5,** 332–334

6 Wilchek, M and Bayer, E A. (1988) The avidin-biotin complex in bioanalytical applications *Anal Biochem* **171,** 1–32

7 Hull, R. and Al-Hakim, A. (1988) Nucleic acid hybridization in plant virus diagnosis and characterization *Trends Biotech* **6,** 213–218

8. Langer, P. R., Waldrop, A A , and Ward, D. C. (1981) Enzymatic synthesis of biotin-labeled polynucleotides· novel nucleic acid affinity probes. *Proc Natl Acad Sci USA* **78,** 6633–6637

9 Miller, S A and Martin, R R (1988) Molecular diagnosis of plant disease *Ann Rev Phytopathol* **26,** 409–432

10 Sambrook, J , Fritsch, E F , and Maniatis, T (1989) *Molecular Cloning A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

11 Heer, A H , Keyszer, G M , Gay, R E , and Gay, S (1994) Inhibition of RNA polymerases by digoxigenin-labeled UTP *BioTechniques* **16,** 54,55

12 Más, P., Sánchez-Navarro, J A , Sánchez-Pina, M A , and Pallás, V (1993) Chemiluminescent and colorigenic detection of cherry leaf roll virus with digoxigenin-labeled RNA probes *J Virol Methods* **45,** 93–102

13 Sánchez-Navarro, J A , Cano, E A , and Pallás, V (1996) Non-radioactive molecular hybridization detection of carnation mottle virus in infected carnations and its comparison to serological and biological techniques *Plant Pathol* **45,** 375–382

# 48

# Detection and Quantification of Plant Viruses by PCR

**Susan Seal and David Coates**

## 1. Introduction

The polymerase chain reaction (PCR) is a technique that enables the specific amplification and hence detection of target DNA sequences from complex mixtures of nucleic acid. A combination of short, specific primers and thermostable DNA polymerases are used to amplify the target sequence, through repeated cycles of denaturation, reannealing, and DNA synthesis at high temperatures, allowing an exponential increase in the amount of the DNA of interest By addition of a reverse-transcription (RT) step, PCR can also be applied to cDNA generated from RNA templates Its extreme sensitivity and high specificity make it an unparalleled technique for the detection and characterization of rare messages, including viral infections that are difficult to detect and diagnose by serology or electron microscopy

Although PCR is now a routine technique in many laboratories, there are still a considerable number of problems in getting good, reproducible amplifications. With plant tissues, the vast majority of the problems have to do with the initial purification of the nucleic acid, to give samples pure enough to be used in the enzymatic reactions. The protocols described here are a combination of routine methods used in our laboratories, and hints and comments on the problems that might be encountered, with pointers to further reading for those working with particularly recalcitrant tissues.

## 2. Materials

### 2.1. General Equipment

1. Polythene bags: 10 × 15 cm, 500 gage (e.g., from Polybags, Greenford, UK).
2. Hand-held wallpaper seam roller narrow roller ~3-cm width (available from hardware stores)

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

3. Microcentrifuge tubes. 2, 1.5, and 0 2–0 5 mL, to fit PCR machine.
4. Micropipetors, adjustable volume from 0.1 to 20 µL, 20 to 200 µL, and 200 to 1000 µL. It is wise to use a separate set solely for pipeting RT and PCR reagents, to reduce chances of crosscontamination.
5. Pipet tips for micropipetors Filter tips further reduce the chance of crosscontamination for pipeting RNA and DNA extracts

## 2.2. Solutions and Reagents

For detection of RNA viruses, considerable care must be taken to limit exposure of extracted RNA to active RNases. Materials and solutions should be treated as described in detail in Sambrook et al. *(1)*, which can be summarized as follows: bake tools and containers at 180ºC for >2 h (if heat-resistant); wearing protective clothing, treat autoclavable containers and solutions with 0.1% (v/v) diethyl pyrocarbonate (DEPC) overnight in fume hood, followed by autoclaving, because RNases cannot be removed from Tris-containing buffers with DEPC, prepare Tris-solutions from RNase-free Tris with DEPC-treated sterilized water and glassware; disposable plasticware (gloves, microcentrifuge tubes, tips, and so on) is usually RNase-free when supplied by manufacturers, and hence need not be treated, but should at all times be handled wearing clean gloves

1 Acid-phenol choroform (5·1 [v/v]) Made from stocks or bought commercially Store in a dark bottle at 4ºC
2 Chloroform. **Caution**—Highly toxic, avoid inhalation. Wear protective clothing, and only carry out manipulations in a fume hood. Store in the dark at room temperature
3 Chloroform·isoamyl alcohol (24 1 [v/v]). Toxic *See* chloroform
4 CTAB extraction buffer. Dissolve 0 4 g cetyltrimethylammonium bromide (CTAB) in 2 mL 1*M* Tris-HCl, pH 8.0, 0 8 mL 0 5*M* EDTA, pH 8 0, 5 6 mL 5*M* NaCl, 5 mL sterile $ddH_2O$. Make up to 20 mL with sterile $ddH_2O$, autoclave at 10 psi for 15 min, and store at room temperature
5 Distilled deionized water ($ddH_2O$)
6 Diethylpyrocarbonate (DEPC). **Caution:** Because this is a mutagen, wear gloves and treat solutions and glass/metalware in a fume hood
7 DNA markers 100 bp ladder (1 µg/10 µL) Mix the following. 190 µL sterile $dH_2O$, 40 µL 6X orange G loading dye, and 10 µL 100 bp ladder stock (1 µg/µL, e.g , Pharmacia 27-4001-01) Store in refrigerator or freezer
8 DNA polymerase 5 U/µL, e g , *Taq* polymerase (Promega M1865) Store at –20°C
9. dNTP mix. 10 m*M*: 10 m*M* of dATP, dCTP, dGTP, and dTTP in sterile $ddH_2O$ Store at –20°C
10. dNTP stocks. 100 m*M* deoxynucleotide triphosphates stocks (HPLC grade) Commercially available

11 1*M* DTT Dissolve dithiothreitol in 10 m*M* Na-acetate, pH 5.5. Sterilize by filtration, dispense into suitable aliquots and store at –20°C
12 0 5*M* EDTA, pH 8.0. Using a magnetic stirrer, suspend $Na_2$ EDTA salt in $ddH_2O$ It will be necessary to adjust the pH to 8 0 by adding NaOH pellets (~20 g) to allow the salt to go into solution Sterilize by autoclaving and store at room temperature
13 Ethanol, 70%. Dilute 95% ethanol with sterile $dH_2O$, and store at –20°C
14 Ethanol Store at room temperature.
15 Ethidium bromide stock (10 mg/mL)· **Caution**—Wearing gloves, dissolve one 100-mg ethidium bromide tablet (Sigma E2515) in 10 mL sterile $ddH_2O$ Store in the dark at room temperature
16 Guanidinium extraction buffer (**Caution:** Toxic, wear gloves) 4*M* guanidinium thiocyanate, 20 m*M* 2-(*N*-morpholino)-ethanesulfonic acid, pH 7 0, 20 m*M* EDTA, 50 m*M* β-mercaptoethanol (add just before use), prepared in sterile $ddH_2O$ **Caution:** Carry out all manipulations involving β-mercaptoethanol in a fume hood, because it is highly toxic and a possible mutagen
17 High-vacuum silicon grease (Dow Corning, Munchen, Germany) Dispense grease into autoclavable polypropylene syringe, wrap in foil, and autoclave Store at room temperature
18 Loading buffer 6X orange G loading dye: Mix 0.9 g Ficoll 400, 0 015 g orange G, 400 µL 0 5*M* EDTA, pH 8 0, and 4 5 mL sterile $dH_2O$ Store at room temperature
19 25 m*M* $MgCl_2$· This is usually supplied with the DNA polymerase obtained commercially Dissolve $MgCl_2$ $6H_2O$ in $ddH_2O$, and autoclave If anhydrous $MgCl_2$ is used, care should be taken when adding $ddH_2O$, because it is extremely hygroscopic Store at room temperature
20 Mineral oil, light white (e g , Sigma M3516)
21 NA extraction buffer 2% (w/v) SDS, 0 1*M* Tris-HCl, pH 8 0, 2 m*M* EDTA, pH 8 0, made from 10% (w/v) SDS, 1*M* Tris-HCl, pH 8 0, and 0 5*M* EDTA, pH 8 0, stocks Autoclave and store at room temperature
22. $Oligo(dT)_{25}$ Dynabeads (Dynal, Oslo, Norway)
23. PCR buffer (10X): An optimum buffer is generally supplied by commercial suppliers for their DNA polymerase
24. PCR primers· 18–30 bases long, 10–100 pmol/µL in sterile $ddH_2O$
25. Phenol **Caution**—All phenol and chloroform-containing solutions are highly toxic, handle in fume hood wearing protective clothing, gloves, and face-shield Do not use phenol solutions that have oxidized and have changed from colorless to light pink. It is recommended to add 0 1% (w/v) 8-hydroxyquinoline to phenol as an antioxidant, a partial inhibitor of RNase, and to chelate metal ions involved in binding RNA to proteins The addition results in the solution turning yellow, and oxidation being visible by a color change to brown Dispose of phenol and chloroform waste according to laboratory regulations
26 Phenol, acid (for RNA viruses) saturated with 0 1*M* citrate buffer ~pH 4 5 (e g , Sigma P4682). Store in a dark bottle at 4ºC
27 Phenol, neutral (for DNA viruses) saturated with TE (e g , Sigma P4557) Store in a dark bottle at 4ºC.

28 Phenol:chloroform.isoamyl alcohol (25:24·1 [v/v]) made from stocks or bought commercially Store in a dark bottle at 4ºC
29 Polyvinyl pyrrolidone (PVP, mol wt 40,000), 20% (w/v)· Prepare fresh on day of use in sterile $dH_2O$
30 Reverse transcriptase, e g , Avian Myeloblastosis Virus (AMV) Reverse Transcriptase at 20–25 U/µL (e g , Promega M9004)
31 RNase A Dissolve at 10 mg/mL in sterile $ddH_2O$ Heat to 95ºC in a water bath for 5 min, then allow to cool to room temperature Dispense and store at –20°C
32 RNasin (40 U/µL, Promega), store at –20°C
33 RT buffer (5X) This is usually supplied with the reverse transcriptase, and, for AMV-RT from Promega, the 5X buffer composition is 250 m*M* Tris-HCl, pH 8 3, 250 m*M* KCl, 50 m*M* $MgCl_2$, 2 5 m*M* spermidine, 50 m*M* DTT
34 RT primers, random hexamers, oligo(dT), or specific downstream primer constructed or obtained commercially. Dilute stock solution to a working concentration of 100 µ*M* for random hexamers, or 50 µ*M* for oligo(dT) or specific primer Store in aliquots at –20°C
35 Sterile $ddH_2O$ Distilled deionized water sterilized by autoclaving
36 sodium dodecyl sulfate (SDS), 10% (w/v) Weigh out in fume hood, and dissolve in warm sterile $ddH_2O$ Store at room temperature, and heat to ~60ºC if detergent comes out of solution subsequently
37 Sephadex G-50 resin· Suspend 10 g of Sephadex G-50 (medium grade) in 160 mL of RNase-free sterile $ddH_2O$ Allow to settle, then pipet off the supernatant and wash the swollen resin three times with RNase-free sterile $ddH_2O$ Equilibrate the resin in 50 mL RNase-free TE and autoclave at 10 psi for 15 min Store at room temperature
38 3*M* Sodium actetate, pH 5 5
39 T7 polymerase (~10 U/µL) and reaction buffer
40 TBE buffer (5X) 0.45*M* Tris-borate, 10m*M* $Na_2$ EDTA For 1 L of 5X stock, dissolve 54 g Tris base, 27 5 g boric acid, and 20 mL $Na_2$EDTA pH 8 0 in $dH_2O$, adjust to 1 L, and store at room temperature.
41 TE 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8 0 Make from 1*M* Tris-HCl and 0 5*M* EDTA, pH 8.0, stock solutions and autoclave. Store at room temperature
42. 1*M* Tris-HCl, pH 8.0 Dissolve Tris base in 900 mL $dH_2O$, and adjust to pH 8 0 (at room temperature) by adding concentrated HCl (~40 mL) in fume cupboard Ensure that the pH electrode is suitable for measuring the pH of Tris-containing solutions accurately Add $ddH_2O$ to make 1 L of solution, and autoclave. Store at room temperature.

## 3. Methods

### 3.1. Preparation of Template

There are many methods for RNA or DNA extraction, and the method of choice will depend on virus and plant tissue. Below is one method for total nucleic acid extraction, one method for DNA extraction from recalcitrant tis-

sue, and two methods for extraction of RNA using a RNase-inactivating extraction buffer. Degradation of RNAs can also be minimized by working rapidly and performing operations at 0–4°C whenever possible.

### *3.1.1 Extraction and Purification of Total Nucleic Acid* (2)

1 Place plant tissue (approx 1 $cm^2$) in a small polythene bag and freeze in liquid nitrogen Grind sample to a fine powder using the seam roller (*see* **Note 1**) As the leaf powder thaws, add 300 µL of nucleic acid extraction buffer, homogenize sample, working quickly, and transfer it to a 1 5-mL microcentrifuge tube (*see* **Note 5**)
2 Immediately add 150 µL of phenol (acid phenol for RNA viruses, neutral phenol for DNA viruses) and 150 µL of chloroform Vortex to mix thoroughly
3. For isolation of RNA, incubate at 70°C for 5 min
4 Centrifuge at room temperature (12,000*g*, 10 min), and transfer 200 µL of the supernatant to a sterile microcentrifuge tube (*see* **Note 2**) For RNA viruses, add 2 µL of 1*M* DTT and 1 µL of RNasin as ribonuclease inhibitors Quick-freeze RNA templates in liquid nitrogen before and after column purification, to minimize RNase activity, and store at –80°C
5 Fractionate the supernatant on a 1-mL Sephadex G-50 column (*see* **Subheading 3.1.2.**), or purify polyadenylated RNAs using oligo$(dT)_{25}$ Dynabeads (Dynal, Oslow, Norway) (*see* **Subheading 3.1.3.**).

### *3.1.2. Sephadex G-50 Column Purification*

1 Plug the bottom couple of mm of a 1-mL disposable syringe with sterile glass wool. Add 1 mL of equilibrated Sephadex G-50 resin to the plugged syringe, and then insert this into a 15-mL centrifuge tube and centrifuge (700*g*, 4 min)
2 Add more resin until the packed column volume is approx 0.9 mL Wash the column twice by adding 100 µL TE and recentrifuging (700*g*, 4 min)
3. Apply 100 µL of thawed nucleic acid to the column; put a sterile microcentrifuge tube under the column before recentrifuging (700*g*, 4 min) to collect the purified extract.

### *3.1.3. Dynabeads Oligo$(dT)_{25}$ Purification of Polyadenylated RNA*

Extract polyadenylated RNAs from 100 µL of the supernatant according to manufacturer's conditions, with the modification that hybridization of RNA to the magnetic beads is carried out for 10 min at room temperature.

### *3 1.4. DNA Purification*

This method was developed to extract DNA from dried fig leaves (the usual methods produce a small brown, latex-like ball), and optimized by M Cornell, D. MacGregor, and P. Gaunt. It is loosely based on the Rogers and Bendich CTAB nucleic acid extraction method *(3)*, followed by purification using DNA-binding resins. Magic Minipreps™ from Promega (Madison, WI) were used

successfully by David Coates' laboratory, but the method should work with any of the column–resin combinations available for purifying plasmid DNA.

1 Freeze 0 5 g leaf material in liquid nitrogen, then grind it in liquid nitrogen in a precooled mortar and pestle
2. Add the powder to 4 mL CTAB extraction buffer prewarmed to 65ºC (for dried material, dilute 1.1 with sterile $ddH_2O$ first), containing 1% (w/v) PVP. Add 100 µL 10 mg/mL RNase A and incubate at 65ºC for 30 min
3 Add 4 mL chloroform/isoamyl alcohol (24 1), mix gently for 5 min Spin for 5 min (benchtop centrifuge), and collect 1 mL of the aqueous phase
4 Extract once more with 1 mL chloroform/isoamyl alcohol, and collect 0 5 mL of the upper phase.
5 Add 1 mL of your favorite DNA-binding resin, mix, and spin for 1 min Decant, resuspend pellet in wash buffer, and load it into the column
6 Wash the column with 2 mL of the appropriate column wash (*see* manufacturer's instructions), spin briefly to dry the resin, then elute with 50 µL of TE pre-heated to 65ºC

### *3 1 5. Guanidinium Extraction Method for RNA*

The method below is Richard Mumford's modification of the method by Logemann et al. ***(4)*** (*see* **Note 6**)

1 Place plant tissue (approx 0 2 g) in a small polythene bag and freeze in liquid nitrogen. Grind sample to a fine powder using the seam roller, and then add 2 vol (400 µL) guanidinium extraction buffer and 20 µL of fresh 20% (w/v) PVP
2 Homogenize sample fully, then transfer supernatant to a sterile microcentrifuge tube containing ~30 µL of sterile high vacuum silicon grease, and an equal sample volume (400 µL) of acid-phenol·choroform (5 1 [v/v]) Vortex sample to form an emulsion
3 Just before centrifugation, add 600 µL DEPC-treated sterile $ddH_2O$ to each sample and then centrifuge (12,000g, 15 min). This reduces the density of the aqueous phase, so that the silicon grease forms a barrier between the organic and aqueous phase
4 Collect supernatant, transfer it to a 2-mL microcentrifuge tube, and precipitate RNA by adding 1 vol of ice-cold isopropanol and incubating at –70ºC for at least 1 h
5 Centrifuge (12,000*g*, 15 min), and decant supernatant, taking care not to lose the RNA pellet Wash pellet with 500 µL cold 70% ethanol, and centrifuge for 3 min (*see* **Note 3**) Remove as much of the supernatant as possible, and dry pellet in a vacuum desiccator or laminar flow cabinet
6. Dissolve dry pellet in 50 µL DEPC-treated sterile $ddH_2O$ (*see* **Note 4**)

### *3.1.6. Guanidinium Extraction Method for RNA (II)*

This is a variation adapted from that used by S. Gurr (personal communication).

1. Precool a small pestle and mortar with liquid nitrogen, and snap-freeze 200–400 mg of plant tissue in liquid nitrogen in a polypropylene tube Make sure there is a pool of liquid nitrogen in the pestle, then hit the tube containing the sample against the bench to fragment the tissue, pour it into the liquid nitrogen in the pestle, and grind to a fine powder. Keep the powder wet all the time by adding more liquid nitrogen, as appropriate.
2. Mix 0.5 mL guanidinium buffer and 0 5 mL acid phenol chloroform·isoamyl alcohol (25:24:1) in an 11-mL polypropylene push-cap tube
3 Let the nitrogen evaporate from the pestle (wait until the tissue changes from dark green to light green), then pour the powder straight into the extraction buffer. Vortex to mix
4 Centrifuge (10 min, 1000*g*) in a bench top centrifuge
5 Transfer the aqueous (top) phase to a 1.5-mL Eppendorf tube containing 0 5 mL phenol:chloroform:isoamyl alcohol Mix, spin, and repeat until the interface is clear (usually three or four times).
6. Collect the aqueous phase, add 0 2 vol of 1*M* acetic acid and 0 7 vol of cold 96% ethanol. Mix and incubate overnight at –20°C.
7 Pellet by spinning at maximum speed (12,000*g*) in a microcentrifuge at 4ºC for 10 min Drain the pellet, add 400 µL 3*M* sodium acetate, pH 5 5, and vortex Respin for 10 min at 4ºC Repeat once more (low-mol-wt RNA and contaminating polysaccharides will redissolve)
8 Remove the salt with a final wash with 70% ethanol, spin 10 min and redissolve the dried pellet in 20–30 µL sterile $ddH_2O$ If hard to dissolve, add more water and/or heat to 95ºC for 2 min and quench on ice.

For hints and comments on recalcitrant tissue, *see* **Note 7**

## 3.2. Design of Primers

Primer design has been discussed extensively in various books (e.g., **refs. *5*** and ***6***), and there are a variety of computer programs that aim to automate the process. In our experience, these programs may be useful, but are not prerequisites to good primer design. There is still too little known about the interactions between short oligonucleotides to be able to produce good predictions on whether a particular primer pair is a good one. In general, the trick is to test several sets of primers, rather than to rely on one set and try to optimize conditions for that pair. With that in mind, the following guidelines are offered.

1 There are no definite rules to guarantee the success of a primer pair Because some primer pairs are 100- to 1000-fold more sensitive than others for elusive reasons ***(7)***, several sets of primers should be tried out
2 Design the primers by visual inspection of the nucleic acid sequence Then, if available, use a primer design program (e.g., primer from the Whitehead Institute) to check for some of the features mentioned below.

3 The primers should be 18–30 nucleotides long, with a $T_m$ of 55°C or above The $T_m$ can be approximated by assuming each A or T contributes 2°C and each G or C contributes 4°C, and adding up the values Where Inosine is used (as a totally redundant base), it will not contribute to the $T_m$ Where redundant primers are used, calculate the lowest $T_m$ and start from there
4 As far as possible, design primers with the same $T_m$
5 Primers should be approx 200–2000 bases apart, if less, the product can be more easily confused with primer-dimers, and, if larger, the product may not be efficiently amplified.
6 Primers forming hairpin loops, or pairs having complementarity at 3' ends should be avoided
7 Do not use oligo(dT) on its own· Have this anchored, by using a mix of 3 oligo(dT) primers with A, C, or G at 3' end (*see* **Note 8**)
8. Mismatching of T bases in target sequence or primer occurs at a higher rate than for other bases, so it is best to avoid a T at the 3' end of the primer for discriminating similar DNAs *(8)* In most cases, try to ensure that the 3' end has a GC clamp
9 Avoid runs of three or more of the same base

Notwithstanding these comments, primer unpredictability is such that, if there is no other choice, try it anyway. A final comment· Check the primer sequences against the nucleic acid databases to make sure you have not inadvertently designed a primer that would amplify some other known sequence.

### 3.3. Reverse Transcription

1 Prepare a master mix of all the components listed below, except the RNA template Make sufficient mix for all samples, plus available positive and negative controls, plus one spare reaction to allow for pipeting errors

For 1 × 25-µL reaction (15 µL mix, 10 µL RNA). 4 µL sterile dd$H_2O$ (DEPC-treated), 5 µL AMV RT buffer (5X), 2.5 µL dNTP mix (10 m*M*), 2 µL downstream primer (50 µ*M*); or 2 µL random hexamers (100 µ*M*), 0 5 µL RNasin (40 U/µL), 1 µL AMV RT (20 U/µL) (*see* **Note 9**).

2 Heat RNA samples (0 05–1 µg in 10 µL vol) at 70°C for 3 min and chill on ice (*see* **Note 10**) Spin samples for a few seconds to remove condensation before adding 15 µL master mix Perform RT at 42°C (37°C for random hexamers) for 30–60 min
3. Because reverse transcriptase activity inhibits *Taq* polymerase, heat-inactivate samples (95°C, 5 min) after RT, then chill on ice

### 3.4. Polymerase Chain Reaction (see Note 11)

The great sensitivity of PCR results in crosscontamination being a common occurrence. Extreme care should be taken with template preparation and setting up of RT and PCR, particularly at steps where aerosols can be formed, e.g., opening of tubes and ejecting tips used for pipeting PCR products (*see* **Note 12**)

1 Prepare a master mix of all the components listed below, except the (c)DNA template. Make sufficient mix for the total number of samples, plus one positive control, two water controls, and one spare reaction to allow for pipeting errors (*see* **Note 13**)

   For 1 × 50-µL reaction 37 8 µL sterile $ddH_2O$, 1.0 µL dNTPs (10 m*M*) (*see* **Note 14**), 5 0 µL PCR buffer (10X), 3 0 µL $MgCl_2$ (25 m*M*) (*see* **Note 15**), 0.5 µL forward primer (50 µ*M*), 0 5 µL reverse primer (50 µ*M*), 0 2 µL *Taq* polymerase (5 U/µL) (*see* **Note 16**), 2 0 µL cDNA template

2. Put 48 µL of mix into PCR tubes, and add 2 µL of DNA template If required, add sufficient mineral oil (1 drop/50 µL) to cover each reaction
3 PCR-amplify by placing in thermocycler, programmed as follows (*see* **Note 17**)·
   a 1 cycle. 95ºC for 2–3 min (initial denaturation)
   b 30–45 cycles· 94°C for 10–60 s (denaturation), 45–70°C for 30 s (annealing), 72–74°C for 30–60 s (extension).
   c 1 cycle 72–74 C for 5 min (final extension).
4. Store samples in refrigerator or freezer till ready for electrophoresis

### *3.4.1. Detection of PCR Products by Gel Electrophoresis (see **Note 18**)*

1 Dissolve 1–2% (w/v) agarose in 0 5X TBE buffer by heating, and cool to ~50–60°C before pouring gel on level surface.
2 Once set, load 5–15 µL of PCR reaction plus 1–3 µL 6X loading dye into each well, and electrophorese samples at 4–10 V/cm distance between electrodes for desired length of time
3 Visualize DNA bands on UV transilluminator after staining for 30 min in dilute ethidium bromide solution (0.5 µg/mL) (*see* **Note 19**)

## *3.5. Quantification of Product*

PCR will be exponential under optimum primer, template, and reaction buffer conditions. However, because of the presence and generation of polymerase inhibitors, suboptimal cycling parameters, degradation in polymerase activity, and unsuitable template:primer ratios, the amplification efficiency is often not exponential, particularly after 15–20 PCR cycles Thus, to discriminate between true and false negatives, and for quantification purposes, the reaction requires the inclusion of a known amount of amplifiable template in each tube as an internal control from which the amplification efficiency can be determined.

The internal control template should be amplified at the same rate as the target, because small differences in amplification will result in large differences in product yields. However, if its sequence is too similar, heteroduplexes can form in later cycles and interfere with amplification. The most important factor controlling the amplification rate is usually the primer sequences *(7)*, if G/C content and the presence of secondary structure are similar for control and

target templates *(9)* The use of the same primer annealing sequences in target and control template is recommended, therefore, and is termed competitive PCR. To quantify the target template, known molar amounts of control template are added into series of reaction tubes containing a set volume of the target template. Target and control products must be distinguishable by size (if necessary, after restriction digestion) or specific hybridization. The amount of control template giving the same yield of product represents the amount of target template.

The advantage of using competitive PCR is that quantification is possible even after less than exponential amplification. If the amplification efficiencies are the same for target and control template throughout the PCR cycles, then the amount of initial template can be calculated by comparison with the yield from a known amount of control template, and use of the equation $X = I(1 + E)^n$, where $X$ = final quantity of product, $I$ = amount of starting template, $E$ = amplification efficiency (maximum value of 1), $n$ = number of cycles (*see* **ref. *10***)

### 3.5 1. Construction of an Internal Control

Considerable care must be taken to find an internal control that is amplified at the same rate as the target, but does not hinder exponential amplification by allowing heteroduplex formation. Moreover, for RNA viruses, because reverse transcription (RT) is rarely 100% efficient and RNases may degrade some of the target during RNA extraction, there is a need for an internal RNA control, which is added to the sample before or during extraction

### 3.5.2. Construction of Control DNA Template

1 Choose a control template for amplification of similar G/C content and size (within 200 bp) as the target, yet easily differentiated by gel electrophoresis
2 Carry out 30 specific amplification cycles of the chosen control sequence with primers for that template that have been modified by addition of the target template primer sequences at their 5' ends
3 Precipitate PCR product by adding 0.1 vol 3*M* sodium acetate, pH 5 5, and 2 5 vol 95% ethanol Incubate at –70°C for >30 min, centrifuge (12,000*g*, 15 min), wash pellet in 70% ethanol, dry, and resuspend in 50 µL sterile $ddH_2O$
4 Reamplify 2 µL of precipitated template with the target template primers and precipitate, wash, and resuspend, as in **step 3**. Run 3 µL of product on an agarose gel to check for single product of desired size, and quantify concentration by UV spectroscopy (260 nm) or a fluorimeter (e g , Hoechst model TKO 100)
5 Check whether the amplification efficiency of the control template equals that of the target template. Add equal copy numbers of control and target template to replicate 50 µL PCR reaction mixes, and remove 5-µL samples of the reaction mixes after 15, 20, 25, 30, and 35 cycles Electrophorese on an agarose gel and

determine yields of control and target template by scanning ethidium bromide-stained gels using a suitable densitometer, or by using computer imaging of the Polaroid film. Plot the increase of the log of the yields of both templates in each tube during the PCR. Equal amplification efficiencies will generate graph lines of the same gradient, and identical product yields, if identical amounts of template were originally added

### *3.5.3. Construction of Control RNA Template*

1. Following a similar procedure to **steps 1–3** in **Subheading 3.5.2.**, incorporate a RNA polymerase recognition sequence, such as 5'-AATTTAATACGACTCACTATAGGGAT-3', for T7 polymerase binding and transcription initiation *(11)* at the 5' end of the forward primer, and carry out in vitro transcription
2. Perform in vitro transcription in a 50-µL reaction vol using 25 µL of above template prepared with modified primers, and 25 µL transcription reaction mix, according to manufacturer's instructions For T7 polymerase (Promega), the final reaction buffer should be 40 m*M* Tris-HCl, pH 7.9, 6 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* NaCl, 10 m*M* DTT, 0 5 m*M* of each ribonucleotide (e g , Promega P1221), 10 U of T7 RNA polymerase, and 20 U of RNasin (e g , Promega N2511) Incubate at 37°C for 2 h
3. Quantitate yield of RNA product by measuring the $OD_{260}$ of an aliquot, and using the approximate conversion factor of 1 $OD_{260}$ unit represents 40 µg of RNA/mL

For an alternative method, *see* **Note 20**.

### *3.5.4. Competitive (RT-)PCR*

1. Estimate the approximate range of target template present in the sample of interest. For each sample, carry out replicate (about five) extractions, to which a series of known amounts of competitor RNA or DNA template, covering the estimated range of target template, have been added.
2. Perform (RT-)PCR on all samples, and electrophorese 10–15 µL of PCR product on an agarose gel, as described in **Subheading 3.4.1.** Measure the yields of target and competitor products
3. Plot the yields of target and competitor products for each PCR reaction in the dilution series, after correcting the values to allow for the size difference of the products. Extrapolate from the graph the amount of competitor DNA that gives rise to an equal molar amount of product to that of the target. Depending on the accuracy required, a couple of runs may be necessary to determine the range of competitor template to add to exactly quantify the copies of template

## *3.6. Internal Controls*

An alternative to the optimized controls discussed in **Subheading 3.5.** is to use an endogeneous gene transcript as a control for RT-PCR. This is especially useful when PCR is being used to check the presence or absence of viral sequences, because all that is needed is a second set of primers which give a

product significantly different in size from the predicted viral product. For RT-PCR, it does require that the cDNA synthesis uses a nonspecific primer, i e., oligo(dT) or random primers, rather than specific primers targeted to the viral sequence. Such a control confers several advantages, because it acts as a control for both the cDNA synthesis and the PCR, and distinguishes between true and false negatives, which is important in epidemiological work. Before using such a control, it is vital to check all the primers both singly and in combination with each other, on infected and uninfected samples, to ensure that there is no interference between the primer pairs, i.e., that the control primers do not amplify the target sequence, or that one test and one control primer do not amplify some other, unknown sequence (*see* **Note 21**)

## 4. Notes

1 Plant samples can alternatively be ground in microcentrifuge tubes using plastic grinders or wooden applicator sticks (Fischer Scientific, Pittsburgh, PA), or in a pestle and mortar. The latter must be cleaned extensively between use, and, for DNA viruses, treated with a DNA-degrading compound, such as 10% sodium hypochlorite, to avoid crosscontamination of samples

2 Interphase material may contain RNases and RT-PCR inhibitory substances, and hence care should be taken to avoid pipeting off any of this material Should the interface not form a tight layer, it is recommended to carry out another phenol chloroform extraction on the supernatant To aid separation of the supernatant from the interface material, a physical barrier like high-vacuum silicon grease can be included, as described in **Subheading 3.1.5**

3 For extraction of RNA, it is advisable to break up precipitated RNA during the 70% ethanol wash, and perform more than one wash, if necessary, because material with RNase activity is often trapped within the precipitated RNA

4 If a RNA–DNA precipitate is particularly difficult to dissolve in sterile $ddH_2O$, this is generally either caused by overdrying of the pellet, or by pelleting through centrifugation being excessive

5 SDS releases both RNA and DNA from protein complexes and partially inhibits RNase action. Extraction with phenol-SDS buffers at 60°C will be fairly effective at removing DNA contamination from RNA extractions

6 Guanidinium methods· These RNA extraction methods are particularly effective for tissues high in RNases, because both guanidinium thiocyanate and β-mercaptoethanol inactivate RNases irreversibly

7 A variety of technical modifications have been proposed and tested by many labs around the world RNA isolation from plants has always been a slightly tricky problem The technical approaches need to overcome two major problems. degradation of RNA, hence the use of powerful RNase inhibitors, and inhibitory contaminants, which are a special problem for plant tissue In many cases, PCR inhibitors can be ignored by simply diluting the sample and using the extreme sensitivity of PCR to amplify desired fragments. When target molecules are rare

to start with, or when particularly noxious inhibitors exist, then some kind of extra purification step is needed. Unfortunately, many inhibitors appear to copurify with nucleic acids, and separation can be difficult. There are two main approaches to getting rid of inhibitors: a chemical approach, in which the nucleic acids and inhibitors, e g , polyphenolics, are separated by differential extraction, and a biochemical approach, in which the target is specifically trapped and washed to get rid of the contaminants This latter approach is particularly useful for viruses, because specific capture columns can be made using antibodies to the viral CP, before rupture of the particles for cDNA synthesis and/or PCR on the now pure nucleic acid ***(12,13)***. Various resins and matrixes are available commercially that have been reported to be sufficiently effective at removing particular inhibitors from samples to allow RT-PCR amplification (e g , Magic/Wizard [Promega], Elutip-d columns [Schleicher and Schuell, Germany], Genereleaser [Cambio, UK]) These methods add considerable cost to sample preparation, and purification through Sephadex G-50 columns can be equally effective for removal of some impurities. Copurification of polysaccharides is a common problem when isolating nucleic acids, and the inhibitory effect some have on *Taq* polymerase can be overcome by including Tween-20 (0.5%), DMSO (5%), or polyethylene glycol 400 (5%) in the PCR buffer ***(14)***. Alternatives are to clean the nucleic acid with a 2*M* sodium chloride precipitation ***(15)***, or to use tissue extraction buffers containing substances such as sodium chloride and cetyltrimethylammonium bromide (CTAB) to reduce copurification of complex carbohydrates, or the cation-exchange resin Chelex-100 (Bio-Rad) at 20–80% (w/v) ***(16)*** Inclusion of 0 2–1% (v/v) β-mercaptoethanol and/or 1% (w/v) polyvinyl polypyrolidone is effective at inhibiting oxidation and removing polyphenols Methods have also been developed using triisopropylnaphthalene sulfonic acid and *p*-aminosalicylic acid (NaTINS-pAS) ***(17)***, hot borate at alkaline pH, in combination with PVP and deoxycholate ***(18)***; or taking advantage of the differential solubilities of nucleic acids and contaminants in solvents such as 2-butoxyethanol ***(19)*** A favored method in our laboratory for DNA extraction from difficult plant species is that of Lodhi et al ***(20)***, which combines CTAB, sodium chloride, polyvinyl polypyrrolidone, and β-mercaptoethanol, with the modification that the concentration of EDTA in the extraction buffer is reduced to 10 m*M*. The method would also be suitable for RNA extraction, but the incubation step at 60ºC should be reduced to 5–10 min to reduce RNA degradation. Poulson ***(21)*** reports that CTAB extractions do not generally completely remove polysaccharides and sugar phosphates, which can be achieved by dissolving RNA in 25 m*M* Tris-HCl, pH 8.0, 25 m*M* sodium chloride, and then adding equal volumes of 2.5*M* potassium phosphate, pH 8 0, and 2-methoxyethanol The mixture is shaken vigorously for 2 min, centrifuged 5 min, and the upper organic phase transferred to a fresh tube An equal volume of 1% CTAB is added, the sample incubated on ice for 5 min, and then centrifuged (5000*g*, 5 min). The RNA pellet is then washed three times with 70% ethanol containing 0 2*M* sodium acetate to convert it to the water-soluble sodium salt

8 The use of oligo(dT) primers for RT of polyadenylated viruses generally only gives rise to 2–3 kb cDNAs Random hexamers will generate cDNA from all RNA, and so may be preferrable

9 Avian myeloblastosis virus (AMV) has a higher RNase H activity than Moloney murine leukemia virus (MMLV), and hence is less suitable for generation of long cDNAs. The best enzyme for templates in which secondary structure interferes with transcription would be the thermostable polymerase *rTth* (Perkin-Elmer) The latter can be used both for RT in presence of $MnCl_2$, and for PCR in presence of $MgCl_2$, after chelating out the manganese ion with EGTA Addition of EGTA must be very accurate to avoid different $MgCl_2$ concentrations in different tubes

10 It is important for the detection of double-stranded (ds) RNA to work quickly to avoid renaturation of strands

11. RT and PCR can be performed in the same mix *(2)*, but for difficult or low template numbers it is preferable to carry out RT and PCR separately under optimal reaction conditions for each enzyme.

12 Kwok and Higuchi *(22)* suggested numerous ways to avoid false positives in PCR, such as the use of positive-displacement pipets, separate work areas for handling of PCR products, regular changing of gloves, and the use of uracil primers followed by uracil DNA-glycosylase (UDG) treatment before the first PCR cycle A simple precaution is to treat all tips, tubes and solutions with 10 min of UV, using, e.g , the Amplirad UV irradiation chamber (Genetic Research Instrumentation, Dunmow, Essex, UK).

13 PCR conditions given are meant as a starting point, from which reaction conditions for each different PCR test should be optimized. Specificity of primer annealing is primarily affected by temperature, magnesium ion concentration, and the concentration of primers and DNA polymerase Annealing temperatures are generally between 55 and 72°C, and a good starting point for testing new primers is 5–10°C below the lowest melting temperature ($T_m$) of the primers used Improved specificity of the primers can be achieved by increasing temperature, but care should be taken to avoid temperatures that reduce the amplification efficiency of the target Note that dNTPs also chelate magnesium ions, so dramatic changes in their concentration will have a concomitant effect on the free magnesium concentration One of the commonest reasons for a usually successful PCR to fail is too low a concentration of free magnesium, because of contaminating chelators, e.g , EDTA from extraction buffers, or increased concentrations of dNTPs

14. The concentration of dNTPs should be 20–200 μ*M* for optimal balance between fidelity, specificity, and yield, a concentration above 200 μ*M* will result in increasingly poor fidelity Primer annealing is aided by 50 m*M* KCl, but higher concentrations, or NaCl at 50 m*M*, will inhibit *Taq* activity Commercially supplied PCR buffers usually contain 10–50 m*M* Tris-HCl, pH 8 0–9.0, but this may not be optimum for some reactions, e.g , long-distance PCR works more effectively at 25 m*M* Tris, pH 9 0 Nonionic detergents (0 5% Tween-20, or Triton X-100), gelatin, or BSA are often included to help stabilize the enzyme

15. The concentration of $MgCl_2$ should generally be in the range of 0 5–2.5 m*M*, and, if the DNA solution contains EDTA, this should be taken into consideration, because it will chelate out the magnesium. Primer concentrations are generally in the range 0.1–1 μ*M* (10–100 pmol/50-μL reaction), and annealing should occur within a few seconds It is best to use the minimum amount of primer that does not limit amplification, to avoid nonspecific priming
16. The choice of polymerase depends on the properties desired; e.g., Vent polymerase has 3'–5' proofreading exonuclease activity, thus resulting in a higher fidelity *Taq* polymerase has historically been the most commonly used enzyme, and is generally added at 1–2 U/50-μL reaction, but the units needed are highly dependent on the commercial source Use of too much enzyme can result in nonspecific amplification. The specificity and sensitivity can also be improved by adding the chosen polymerase enzyme once the initial denaturation step has been completed (termed hot-start PCR)
17. The duration of each cycle should also be optimized It is important that complete denaturation of the starter template is achieved, without causing excessive denaturation of the enzyme (*Taq* polymerase has a half life of ~40 min at 95ºC). Increasing the duration of the annealing step will decrease specificity *Taq* polymerase can incorporate about 150 bases/s/enzyme molecule, under optimum conditions. A safe rule is to allow 1 min at 72ºC for every 1 kb of desired product

18 3.1% NuSieve·SeaKem (w/w) agarose (FMC, Rockland, ME) gives better resolution for experiments in which the additional cost can be justified

19 Ethidium bromide staining is the most common method, but, if greater sensitivity is required, it can be coupled with blotting and a hybridization step An alternative is to include a fluorescent-labeled nucleotide during PCR. The greatest sensitivity of detection of fluorescence is by gel electrophoresis on a fluorescent DNA sequencer, which is 100-fold more sensitive than detecting fluorescence on a solid phase ***(23)*** The amount of PCR product can also be quantified by incorporating a radiolabel (e.g., 50 μCi/mL [$-^{32}$P]dCTP) In this case, measurement of particle emission can be carried out over a 2-log larger range than light transmittance, and hence may be preferred ***(24)***

20 Synthesizing an internal RNA standard· Alternatively, clone a section of DNA containing primer sequences into a vector containing T3, T7, or SP6 polymerase recognition sequence, and perform in vitro transcription according to manufacturer's instructions. Because it is often desirable to be able to compare the results from one RT-PCR run with another, ideally, the standard used should be from the same preparation To avoid RNA degradation between experiments, precipitate the RNA in the presence of carrier RNA, and keep suitable aliquots at –80ºC When needed, pellet, redissolve, and quantify the amount by UV spectroscopy

21 Our experience with this technique is with PCR from insects, rather than plants For PCR from DNA, primers against any single copy genomic sequence are worth trying For RT-PCR in aphids, we have used actin as a control; sequences are highly conserved, and expression is essentially constitutive The primers

(designed using the rules in **Subheading 3.2.**) work in all aphid species, and in other insects, e.g., *Myndus*

## References


1 Sambrook, J , Fritsch, E F , and Maniatis, T (1982) *Molecular Cloning A Laboratory Manual*, 2nd ed Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

2 Pappu, S S , Brand, R , Pappu, H R., Rybicki, E P , Gough, K H , Frenkel, M J , and Niblett, C L (1993) A polymerase chain reaction method adapted for selective amplification and cloning of 3' sequences for potyviral genomes: application to dasheen mosaic virus *J Virol Methods* **41,** 9–20

3 Rogers, S O and Bendich, A J (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant-tissues *Plant Mol Biol* **5,** 69–76

4 Logemann, J , Schell J , and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues *Anal Biochem* **163,** 16–20

5. McPherson, M J , Oliver, R O , and Gurr, S. J. (1992) The polymerase chain reaction, in *Molecular Plant Pathology A Practical Approach*, vol 1 (Gurr, S J , McPherson, M J , and Bowles, D. J , eds ), IRL, Oxford, New York, and Toronto, pp 123–146.

6. Taylor, G R (1991) Polymerase chain reaction: basic principles and automation, in *PCR A Practical Approach* (McPherson, M J , Quirke, P , and Taylor, G R , eds ), IRL, Oxford, New York, and Toronto, pp 1–14.

7 He, Q , Marjamaki, M , Soini, H , Mertsola, J , and Viljanen, M (1994) Primers are decisive for sensitivity of PCR *BioTechniques* **17,** 82–87

8 Gelfand, D. H. and White, T J (1990) Thermostable DNA polymerases, in *PCR Protocols A Guide to Methods and Applications* (Innes, M A , ed ), Academic, San Diego and London, pp 129–141

9 Siebert, P. D. and Larrick, J W (1992) Competitive PCR *Nature* **359**, 557–558

10 Gilliland, G., Perrin, S , Blanchard, K , and Bunn, H (1990) Analysis of cytokine mRNA and DNA. detection and quantitation by competitive polymerase chain reaction *Proc Natl Acad Sci USA* **87,** 2725–2729

11 Blais, B W (1994) Transcriptional enhancement of the *Listeria monocytogenes* PCR and simple immunoenzymatic assay of the product using anti-RNA DNA antibodies. *Appl Environ Microbiol* **60,** 348–352.

12. Nolasco, G , de Blas, C , Torres, V , and Ponz, F (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens *J Virol Methods* **45,** 201–218

13 Brandt, S., Himmler, G., and Katinger, H (1993) Application of the immunocapture polymerase chain reaction (IC/PCR) for detection of grapevine viruses in woody material *Mitteilungen Klosterneuburg* **43,** 143–147

14 Demeke, T. and Adams, R P. (1992) The effects of plant polysaccharides and buffer additives on PCR *Biotechniques* **12,** 332–334.

15 Fang, G , Hammar, S , and Grumet, R. (1992) A quick and inexpensive method for removing polysaccharides from plant genomic DNA *Biotechniques* **13,** 52–55

16. Ochert, A S., Boulter, A W , Birnbaum, W., Johnson, N W., and Teo, C G (1994) Inhibitory effect of salivary fluids on PCR· potency and removal *PCR Methods Applic* **3,** 365–368
17 Felipe, M. S S., Rogelin, R , Azevedo, M O , and Astolfifilho, S (1993) Extended application of an efficient method for RNA isolation from different organisms *Biotechniques* **7,** 639–644
18. Wan, C. Y. and Wilkins, T A. (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L). *Anal Biochem* **223,** 7–12
19 Manning, K (1991) Isolation of nucleic acids from plants by differential solvent precipitation. *Anal Biochem* **195,** 45–50.
20 Lodhi, M. A., Ye, G -N , Weeden, N F , and Reisch, B I (1994) A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species *Plant Mol Biol Reporter* **12,** 6–13
21 Poulson R (1973) Isolation, purification an fractionation of RNA, in *The Ribonucleic Acids* (Stewart, P R and Letham, D S.), Springer-Verlag, Berlin, pp 243–261
22 Kwok, S. and Higuchi, R (1989) Avoiding false positives with PCR. *Nature* **339,** 237,238
23 Winn-Deen, E S , Batt, C A , and Wiedmann, M (1993) Non-radioactive detection of *Mycobacterium tuberculosis* LCR products in a microtitre plate format *Mol Cell Probes* **7,** 179–186
24. Ferre, F. (1992) Quantitative or semi-quantative PCR reality versus myth *PCR Methods Applic* **2,** 1–9

# 49

# Assaying Levels of Virus with Local Lesion Hosts

**Neil Boonham and K. Roger Wood**

## 1. Introduction

A number of methods have been used for the quantitation of virus within infected plants. Early ones used physical techniques such as dry-wt and particle counts; more recently, serological and nucleic acid-based methods have been developed. These methods are all based on a physical aspect of the virus particle, and all measure total amounts of virus (or viral component), regardless of infectivity. Holmes *(1)*, however, was the first to utilize the observation that mechanical inoculation of tobacco mosaic virus (TMV) onto the leaves of *Nicotiana glutinosa* led to the formation of local lesions, and that the number of local lesions was inversely correlated to the dilution of the inoculum. The local lesion assay remains the simplest method to quantitatively measure the most important biological property of a sample of virus, that of the presence of viable virus particles.

A number of points should, however, be borne in mind when using an assay of this kind. First, there is the obvious requirement for the availability of an appropriate host, one which responds to mechanical inoculation with the formation of clear necrotic lesions or ringspots, or chlorotic spots (*see* **Fig. 1** and **Table 1**). Second, there is almost always a significant variation in response between plants, and between leaves on the same plant. The experimental design must take this into account. However, care should be taken not to overcomplicate the layout used, since the length of time required to inoculate and the risk of error in such an experimental design may outweigh the advantages gained from the randomization. In addition, although half-leaf comparisons give least variation in lesion number, the difficulty in carrying out many half-leaf comparisons makes the use of whole opposite leaves more practical. Finally, the nature of the curve (*see* **Subheading 3., step 7**;

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

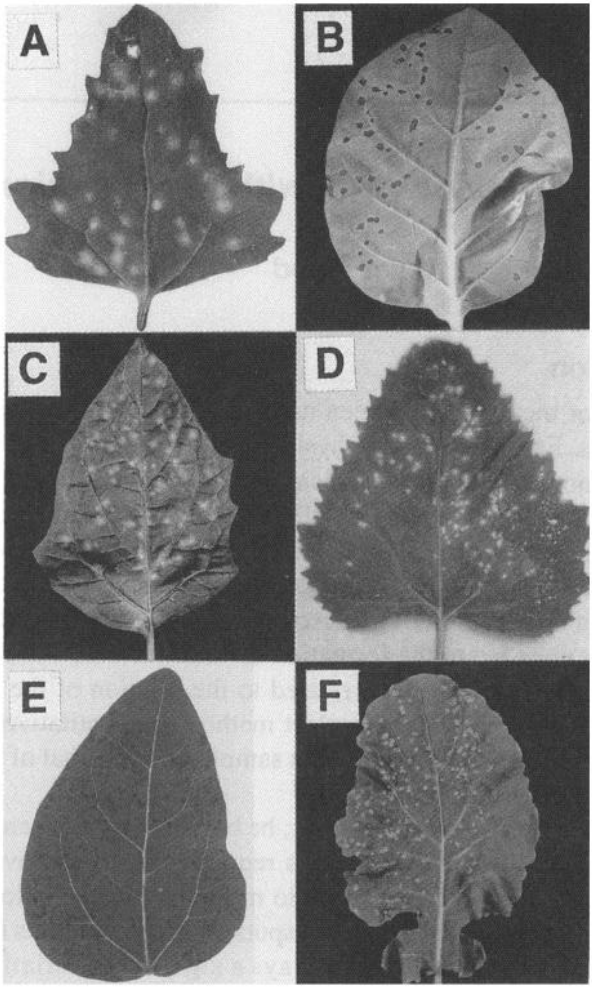



Fig. 1. **(A)** Cucumber mosaic virus/*C. quinoa*. **(B)** Tobacco mosaic virus/*Nicotiana tabacum* cv. Samsun NN (T. M. A. Wilson, SCRI). **(C)** Watercress yellow spot/*Datura stramonium* (Walsh, HRI). **(D)** Beet necrotic yellow vein virus/*C. amaranticolor* (Henry, CSL). **(E)** Cucumber mosaic virus/*Vigna unguiculata* spp. *sinensis*. **(F)** Turnip mosaic virus/Swede (Walsh, HRI).

**Note 2)** relating dilution of inoculum to the number of local lesions should be taken into account, ensuring that comparisons are made within the middle part of the curve, where the number of lesions present is proportional to the dilution of the inoculum.

**Table 1**
**List of Local Lesion Host and Virus Combinations for Type Members of all Virus Groups**

| Genus | Type member | Local lesion host/comments |
|---|---|---|
| *Alfamovirus* | Alfalfa mosaic virus | *Phaseolus vulgaris* and *Vigna unguiculata* spp *sinensis* for most strains, *Chenopodium amaranticolor* and *Chenopodium quinoa* are also suitable |
| *Alphacryptovirus* | White clover cryptic virus 1 | Not mechanically transmissible |
| *Badnavirus* | Commelina yellow mottle virus | Not mechanically transmissible |
| *Betacryptovirus* | White clover cryptic virus 2 | Not mechanically transmissible |
| *Bromovirus* | Brome mosaic virus | *Chenopodium hybridum* and *Datura stramonium* |
| *Bymovirus* | Barley yellow mosaic virus | None |
| *Capillovirus* | Apple stem grooving virus | *P vulgaris* cv Pinto and *C quinoa* |
| *Carlavirus* | Carnation latent virus | *C amaranticolor* and *C quinoa* (difficult to transmit from carnation because of sap inhibitors) |
| *Carmovirus* | Carnation mottle virus | *C amaranticolor* and *C quinoa* (will also detect attenuated strains) |
| *Caulimovirus* | Cauliflower mosaic virus | *Brassica campestris* cv Just Right |
| *Closterovirus* | Beet yellows virus | Difficult to inoculate mechanically |
| *Comovirus* | Cowpea mosaic virus | *P. vulgaris* cvs. Pinto and Scotia, *C amaranticolor* |
| *Cucumovirus* | Cucumber mosaic virus | *Vigna unguiculata* spp sinensis, *P vulgaris*, *C amaranticolor*, and *C quinoa* |
| *Cytorhabdovirus* | Lettuce necrotic yellows virus | *Nicotiana glutinosa* |
| *Dianthovirus* | Carnation ringspot virus | *C. amaranticolor*, *C quinoa*, and *V unguiculata* spp *sinensis* |
| *Enamovirus* | Pea enation mosaic virus | *Chenopodium album*, *C amaranticolor*, and *C quinoa* (album and quinoa are considered to be the most reliable) |

*(continued)*

**Table 1 *(continued)***

| Genus | Type member | Local lesion host/comments |
|---|---|---|
| *Fabavirus* | Broad bean wilt virus 1 | *V unguiculata* spp *sinensis* for the broad bean strain (giving red/brown lesions), *C amaranticolor* and *C quinoa*, for the nasturtium, parsley, and petunia strains |
| *Fijivirus* | Fiji disease virus | Not mechanically transmissible |
| *Furovirus* | Soilborne wheat mosaic virus | *C quinoa* and *C amaranticolor* |
| Subgroup I geminivirus | Maize streak virus | Not mechanically transmissible |
| Subgroup II geminivirus | Beet curly top virus | None |
| Subgroup III geminivirus | Bean golden mosaic virus | *P vulgaris* cv Top crop, gives chlorotic lesions on primary leaves |
| *Hordeivirus* | Barley stripe mosaic *virus* | *C quinoa* and *C amaranticolor* |
| *Idaeovirus* | Rasberry bushy dwarf virus | *P vulgaris* cv The Prince, grown at 20°C and 5000 lux with a 15-h photoperiod Keep the plants in the dark for 1 d prior to inoculation *Chenopodium murale* is reliable, but the lesions are more difficult to count |
| *Ilarvirus* | Tobacco streak virus | *Cyamopsis tetragonoloba*, *V unguiculata* spp *cylindrica*, *Beta patellaris*, *Dolichos biflorus*, *Gomphrena globosa*, and *P vulgaris* cv Monteiga |
| *Luteovirus* | Barley yellow dwarf virus | Not mechanically transmissible |
| *Machlomovirus* | Maize chlorotic mottle virus | None |
| *Marafivirus* | Maize rayado fino virus | Not mechanically transmissible |
| *Necrovirus* | Tobacco necrosis virus | *P vulgaris* and *C amaranticolor* |
| *Nepovirus* | Tobacco ringspot virus | *V unguiculata* spp. *sinensis*, *Nicotiana tabacum*, *Nicotiana clevelandii*, *C amaranticolor*, and *Cassia occidentalis* |

*(continued)*

**Table 1 *(continued)***

| Genus | Type member | Local lesion host/comments |
|---|---|---|
| *Nucleorhabdovirus* | Potato yellow dwarf virus | *Nicotiana rustica* |
| *Oryzavirus* | Rice ragged stunt virus | Not mechanically transmissible |
| *Phytoreovirus* | Wound tumor virus | None |
| *Potexvirus* | Potato virus X | *Gomphrena globosa* (the middle leaves of a plant with 8–10 leaves are most suitable) |
| *Potyvirus* | Potato virus Y | *C amaranticolor*, *C quinoa*, *Nicotiana repunda*, *N rustica*, *Physalis floridana*, *Solanum tuberosum* cvs Duke of York or Saco, and *Solanum demissum* "Y"; the latter produces local lesions with most strains |
| *Rymovirus* | Ryegrass mosaic virus | None |
| *Sequivirus* | Parsnip yellow fleck virus | *C quinoa* |
| *Sobemovirus* | Southern bean mosaic virus | *Phaseolus aureus* |
| *Tenuivirus* | Rice stripe virus | Not mechanically transmissible |
| *Tobamovirus* | Tobacco mosaic virus | *N glutinosa*, *C amaranticolor*, *P vulgaris* cv Pinto, and *Nicotiana tabacum* cvs Xanthi nc and Samsun NN |
| *Tobravirus* | Tobacco rattle virus | *C amaranticolor* and *P vulgaris* |
| *Tospovirus* | Tomato spotted wilt virus | *Petunia hybrida* cvs. Pink beauty and Minstrel |
| *Tombusvirus* | Tomato bushy stunt virus | *C amaranticolor*, *Datura stramonium*, and *Ocimum bacilium* |
| *Trichovirus* | Apple chlorotic leaf spot | *C quinoa* and *P vulgaris* |
| *Tymovirus* | Turnip yellow mosaic virus | Chinese cabbage. Most strains give chlorotic local lesions; young well-nourished plants must be used |
| *Umbravirus* | Carrot mottle virus | *P vulgaris* cv The Prince, grown at 20°C and 2500 lux with an 8 h photoperiod. *C quinoa* and *N tabacum* cv Xanthi can also be used |
| *Waikavirus* | Rice tungro spherical virus | Not mechanically transmissible |

If experiments are designed taking these factors into account, the assay gives a simple and rapid method for obtaining information, not about total virus concentration, but data regarding the relative concentrations of infective virus particles in the samples compared.

## 2. Materials

1 Grinding buffer, for infected plant material and for dilution of standard virus preparations, generally, 0.05*M* phosphate buffer, pH 8 0.
2 Test plants, as uniform as possible It is advisable to grow more plants than required and to select the most uniform of the group In addition, arrange the sets of plants in order to allow equal numbers of each size group of plants in each sample replicate Plants should be trimmed to leave only the leaves to be inoculated according to the experimental design used
3 Abrasive powder, preferably Carborundum (600 mesh); Celite can also be used
4 A number of 3-cm squares of Parafilm, enough for one piece per inoculation.

## 3. Methods

1 Select the test plants and label the leaves to be inoculated by punching out small disks in the leaf tip, using a glass Pasteur pipet, so that each different inoculum can be identified (*see* **Notes 1** and **6**).
2 Dust the leaves to be inoculated with abrasive powder, to give a light, even covering.
3 Prepare the inoculum by homogenizing the leaf tissue to be tested in a pestle and mortar, on ice in ice-cold grinding buffer (1·1 [w/v]). Serially dilute the stock in the grinding buffer (*see* **Note 2**), keeping all the samples on ice until the inoculations are carried out. Sufficient inoculum is required for ~25-µL inoculations of each dilution per test leaf.
4 Pipet the inoculum onto the leaf and smooth onto its surface, from petiole to tip, using a piece of Parafilm, under even pressure. The leaf must be supported from beneath to prevent damage to the plant. To avoid carryover of inoculum, the Parafilm must be changed for each sample (*see* **Notes 3** and **4**)
5 Immediately following inoculation, spray the leaves of the test plants with water to remove the abrasive powder, to remove sap residues that may inhibit infection or adversely affect the appearance of the leaf, and to help prevent wilting
6. Incubate the plants at 23°C with a 16-h photoperiod, for approx 3–4 d, then count the local lesions present on the leaf surface
7 The tabulated data can be presented graphically, plotting the log of the local lesion number (average of the replicates) vs the log of the dilution From data in the linear part of the graph, it is possible to extrapolate to the intercepts on the *x*- and the *y*-axes, to give both a theoretical dilution end point and an undiluted lesion number, respectively; both are arbitrary measurements, but offer a simple method for making relative comparisons of viable virus concentration among a number of samples (*see* example in **Fig. 2**; **Notes 5** and **7**)

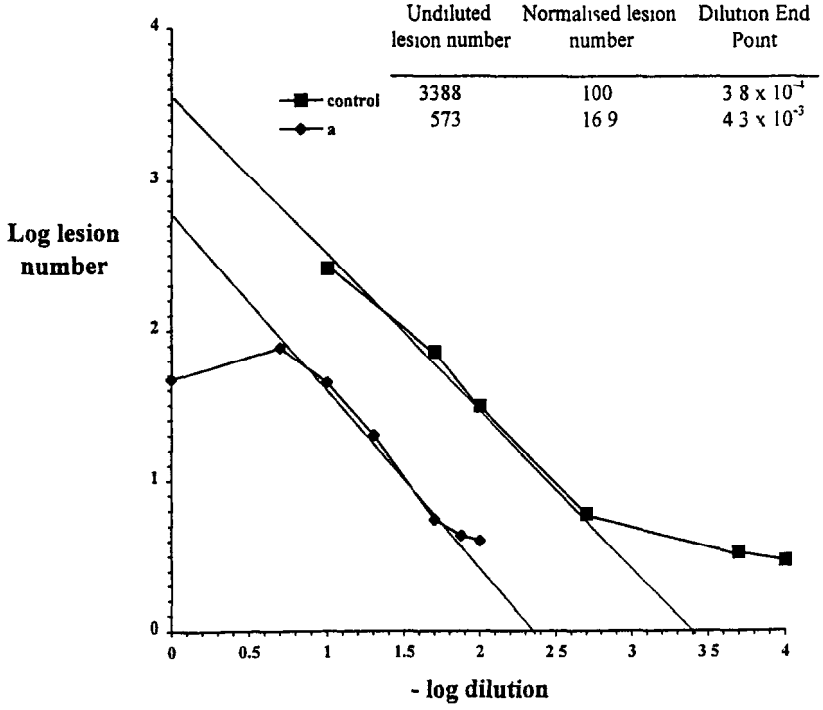



Fig. 2 Graph showing an example of data relating local lesion number to the dilution of inoculum, for CMV inoculated onto cowpea Also included are the calculated lesion numbers for undiluted samples, the normalized lesion numbers, and the dilution end points The dilution end points and the undiluted lesion numbers are calculated as the antilog of the intercept on the *x*- and *y*-axis, respectively.

## 4. Notes

1 The experimental design is of utmost importance to the success of an experiment of this kind The most important factor to take into account is the variability among the plants and leaves inoculated. Leaves of different ages on the same plant do not always react in the same way, and leaves of similar sizes and ages from different plants may not react in the same way. In general, the leaves selected for inoculation should be of similar age and size, and, in most cases, the use of leaves at the same position and on opposite sides of the main stem gives much less variation If experiments are carried out in this way, six to eight replicates for each sample should give sufficient accuracy The design of the experiment depends, then, largely on the number of samples to be compared and the type of the host plant.

The simplest example is one in which only two samples are to be compared, using a host that has leaves opposite to each other, such as cowpea (*Vigna*

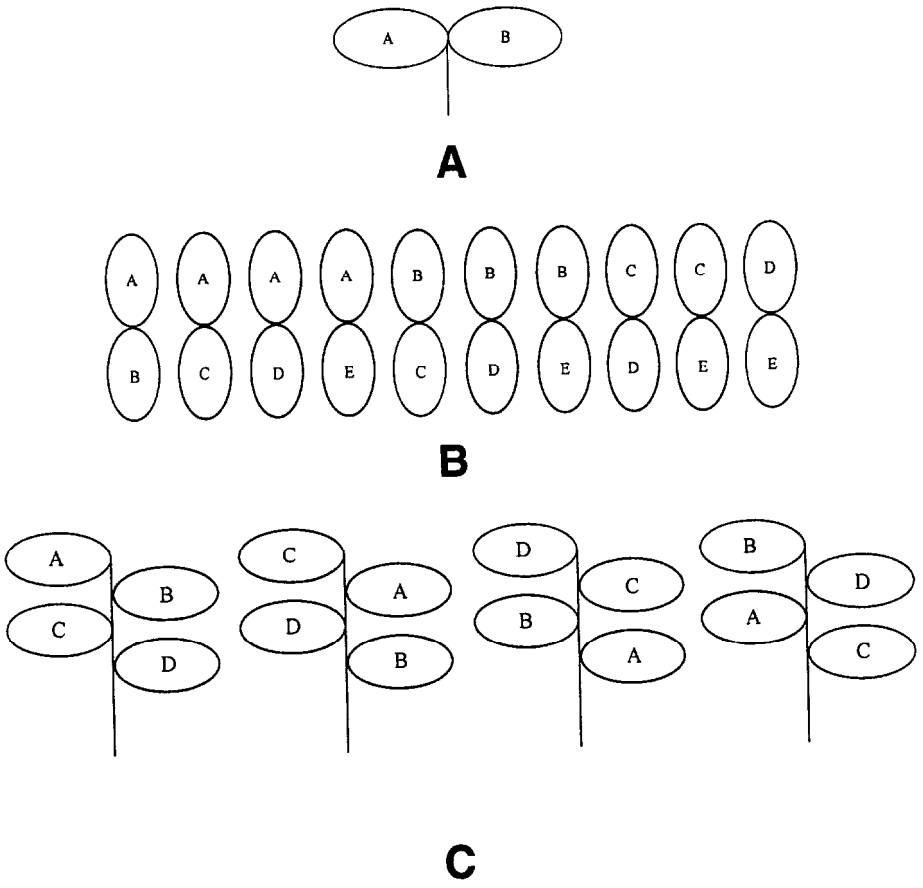



Fig 3 Examples of inoculation patterns used to randomize treatments upon test material for: **(A)** two samples in a direct comparison; **(B)** five samples, where two primary opposite leaves are available for inoculation, and **(C)** a Latin square in which four samples are inoculated to plants with multiple leaves available for inoculation.

*unguiculata* spp. sinensis) In this case, the two samples can be compared on opposite primary leaves The outline of an experiment of this kind is shown in **Fig. 3A**, comparing samples A and B.

If multiple samples are to be compared, again using a host that has opposing leaves, it is suitable to compare each sample with each of the other samples on opposite sides, an equal number of times, as shown in the outline in **Fig. 3B** In this case, samples A–E are being compared

If multiple samples are to be compared using a host in which a number of leaves are available for inoculation, such as *Chenopodium quinoa*, where four to eight leaves may be available, a Latin square design is most suitable In this lay-

out, each sample is compared at each leaf position on the assay plants An example of an experimental outline of this kind is shown in **Fig. 3C**, comparing samples A–D.

In each of these three cases, replication can be achieved by duplication of the sets of plants

2 The dilutions at which the samples are compared are important for a number of reasons. Local lesion curves relating dilution to number of lesions are generally sigmoidal in shape At very high concentrations of virus, a change in concentration has very little change in the number of lesions This may be because of aggregation of virus particles or inhibitors present in the inoculum, at very low concentrations of virus, changes in concentration similarly have little overall effect on the number of lesions present, because of the low efficiency of the mechanical inoculation procedure *(2)* The slope of the curve may also vary, depending on the number of particles required to cause infection However, some virus–host combinations may not respond to give the predicted single- or multiple-hit curves *(3)* To make meaningful comparisons, it is necessary to always compare samples within the middle range of the curve, where a change in concentration is accompanied by an equivalent change in lesion number An example of a local lesion dilution curve, comparing a sample to a standard, is shown in **Fig. 2** It may be necessary to carry out preliminary experiments to reach the desired number of lesions per leaf, followed by an experiment in which the dilutions can be closer together, around the range that gave the desired number of lesions For leaves the size of cowpea, for example, leaf counts in the range of 10–200 local lesions would give useful estimates. It is worth bearing in mind that crude extracts from some plants contain inhibitors of infection, and it may be necessary to dilute the inoculum significantly to obtain adequate lesion numbers

3 As an alternative to pipeting a standard volume of inoculum onto the leaf and spreading it with a Parafilm-coated finger, a small pad of muslin can be soaked in inoculum and rubbed over the leaf surface. In any event, it is important to ensure that the leaf surface is completely wetted with inoculum

4. It is only necessary to rub the inoculum gently over the leaf surface once or twice Repeated rubbing is unnecessary and will lead to unwanted leaf damage

5. If a standard (for example, purified virus) is included in each local lesion assay, the data can be normalized to it and the data from successive experiments can then be compared To normalize the data, use the following equation, setting the standard lesion number to 100 in each experiment

$$\text{Number of lesions produced by undiluted} / \text{Number of lesions produced by the undiluted standard sample} \times 100 = \text{Normalized lesion number}$$

If aliquots of the same standard are used in each experiment, and if multiple freeze–thaw cycles are avoided, data from successive experiments can be compared by using the normalized data

6. **Table 1** lists the local lesion hosts appropriate for the type members of all the genera; if the virus–host combination you require is not in this list, the best

source guide is the CMI AAB *Descriptions of Plant Viruses (4)* For new viruses, the best hosts to try are *Chenopodium amaranticolor*, *C quinoa*, *Vigna unguiculata* spp sinensis, and *Phaseolus vulgaris* If problems are encountered in obtaining the required numbers of local lesions, the response can often be increased by leaving the plants in the dark for 24 h prior to inoculation *(5)*. Age of the plant can also be important. For example, the susceptibility of primary leaves of cowpea to cucumber mosaic virus decreases markedly when trifoliate leaves begin to appear.

7 If it is necessary to make more than just simple comparisons of samples, and if anything other than large differences are sought, the local lesion data requires some statistical analysis, **refs. *6–8*** review the analysis in more detail

## References

1 Holmes, F. O. (1929) Local lesions in tobacco mosaic *Bot Gaz* **87,** 39–55
2 Matthews, R E. F (1991) Methods for assay, detection, and diagnosis, in *Plant Virology* (Matthews, R E F., ed ), Academic, San Diego, pp 11–52
3. Fulton, R W (1962) The effect of dilution on necrotic ringspot virus infectivity and the enhancement of infectivity by non-infective virus *Virology* **18,** 477–485
4. Descriptions of Plant Viruses Commonwealth Mycological Institute, Association of Applied Biologists, Kew, Surrey, England
5 Bawden, F C and Roberts, F. M (1947) The influence of light intensity on the susceptibility of plants to certain viruses. *Ann Appl Biol* **34,** 286–296
6 Kleczkowski, A (1949) The transformation of local lesion counts for statistical analysis. *Ann Appl Biol* **36,** 139–152
7 Kleczkowski, A (1950) Interpreting relationships between concentrations of plant viruses and numbers of local lesions *J Gen Microbiol* **4,** 53–69
8 Roberts, D A (1964) Local-lesion assay of plant viruses, in *Plant Virology* (Corbett, M. K. and Sisler, H. D., eds ), University of Florida Press, Gainesville, pp 194–210

# 50

# Field Testing Resistance of Transgenic Plants

**Wojciech K. Kaniewski and Peter E. Thomas**

## 1. Introduction

This chapter describes methods to detect and assess commercial resistance to virus disease that may be conferred on existing plant cultivars by their transformation with genes derived from viruses *(1)*. The principles and general guidelines for selection among plants altered by transformation are the same as those established for classical breeding *(2)*. However, the approach is different, since transformation alters one or, at most, a few characteristics in an established cultivar; but breeding creates an entirely new cultivar.

A number of factors give rise to the need for field testing of transgenic plants Although it is true that some viral genes will confer transgenic resistance, and this resistance may be added to a cultivar as a single characteristic, it is not true that resistance is achieved in every transgenic plant line. In fact, expression of resistance may be rare among lines transformed with a viral gene, and degree of resistance conferred by a gene can vary continuously from no resistance to complete protection from disease. Furthermore, somoclonal variability in cultivar characteristics may be very common and subtle among transformed plants. Somoclonal variability in virus susceptibility may also occur in the absence of any viral gene *(3)* Therefore, rigorous selection among hundreds of transformants may be required to identify lines that are resistant and that also conform to or exceed standard agronomic characteristics of the parent cultivar. Selection for agronomic performance, described in Chapter 51, should proceed concurrently with resistance selection, described in this chapter.

Field testing required to determine whether interactions occur between the transgene and its homologous virus, or with other viruses when they infect the transgenic crop, is also covered in this chapter.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

## 2. Materials

Analysis of plants for virus infection at intervals during the growing season requires a laboratory equipped and supplied to perform serological and nucleic acid-based analyses. Facilities are also required for propagation of biological vectors of the virus. The vectors are needed both to provide field exposure to the virus by direct inoculation and to perform diagnostic transmissions. Controlled plant growth facilities are required to propagate plants to be tested, to produce index hosts used for virus diagnosis, and to perform diagnostic transmission assays. Analyses to determine whether viruses that replicate in transgenic plants are altered through interactions with the transgene or its products requires a fully equipped molecular biology laboratory.

Requirements for a satisfactory field site, culture, cultivation, and harvest machinery, a computer database, and tissue-culture facilities are the same as those needed to select for agronomic characteristics, and have been described in Chapter 51.

## 3. Methods

### 3.1. Plant Culture

#### 3 1.1 Field Preparation

The standard cultivation and weed and disease control methods used in commerce for the parental cultivar are desirable, since they provide the conditions under which the transgenic cultivar must perform

#### 3.1.2. Propagation of Transgenic and Control Lines

The type of propagants used in field testing will depend largely on whether the crop is grown from true seed or vegetative parts Recommended propagation methods are given in Chapter 51

#### 3.1.3. Growing and Transplanting In Vitro-Produced Plantlets

In vitro-produced plantlets must be transferred to sterile soil or artificial planting medium in small pots, and then transplanted to the field after a period of growth and soil adaptation Recommended methods for transplanting, disease control, fertilization, and development of a strong root system (**Note 1**) are given in Chapter 51

Plantlets should be transplanted from the flats to the field while they are growing vigorously. Transplanting machinery may be used for this purpose, but the process requires meticulous organization to prevent errors when hundreds of small, replicated plots are involved (**Note 2**).

#### 3.1.4. Plant Care During Growing Season

Insect and disease control in plots used to select for resistance to virus disease often presents a special problem, since the treatments used to control

unwanted pests can interfere with the biological vectors needed to provide virus transmission in the current test. In some instances, pesticides may be available that will selectively control the unwanted pests. Good judgment is required to determine the level of insect and disease contamination that can be tolerated in the selection process.

Selection for resistance among many lines frequently requires selective pesticidal treatments to maintain noninoculated control plots and to protect from other diseases. This can be hazardous when done with backpack spray equipment, and it can be difficult to achieve without drift to untreated plots when performed by machine.

Additional methods needed for plant care during the growing season are the same as those required to select for agronomic performance of transgenic lines, and are described in Chapter 51.

### *3.1.5. Harvest and Storage*

Small plot harvesting without mixing between plots and without labeling mistakes is essential, but difficult to achieve. Careful planning and close supervision is essential. Appropriate storage is especially important in cases in which the virus disease affects storability of the crop. It is difficult to achieve when refrigeration, humidity, and control of atmospheric gasses are required.

## *3.2. Classifying Resistance*

### *3.2.1. Terminology Based on Viral Function*

For commerce, a resistant cultivar is one that remains relatively unaffected economically by disease in the field, compared with the susceptible parental cultivar. This concept serves commerce well, but it deals with the crop as a whole. To deal effectively with the ideas involved in development and assessment of transgenic resistance, we need terminology that specifies how and at what stage the normal viral functions required for virus survival are restricted in the individual resistant plant, and we need to consider how these restrictions imposed by the resistant plant may affect epidemiological processes required for virus survival. We also need to understand that it is not virus infection, but rather the severity of host reaction to infection that causes disease loss The terminology proposed by Cooper and Jones *(4)* deals with virus–host interactions in the individual plant and will be used here. In their proposal, a plant is either infectible or immune (not infectible) An infectible plant is classified as susceptible if specific viral functions required for virus survival in the plant proceed with relatively little or no restriction It is classified as resistant if these functions proceed with considerable restriction. The plant is classified as tolerant if its symptom response is mild, or as sensitive if the symptom response

is severe. Symptom response may, but does not necessarily, reflect the degree to which viral functions are restricted

Essential viral functions in the virus–host interaction include initial establishment of infection, multiplication in the initially infected cell, movement of the virus or its message for replication to adjoining cells, and systemic transport throughout the host. Epidemiological processes necessary for a virus to be acquired from one plant and transmitted to another include compatible virus–vector and vector–host interactions. Processes involved in virus–vector interactions may be controlled by viral genes, but those associated with vector–host interactions, including feeding of the vector on the host and deposition of the virus into the plant, are not controlled by viral genes and should not be affected by any pathogen-derived transgenic resistance

### *3 2 2 Elucidation of Viral Function Involved in Resistance*

The kind of field resistance expressed may depend on the function repressed Reduced incidence of disease, for example, may reflect repression of the initial establishment of infection or failure of virus to move from initially infected cells. Resistance to spread of virus from plant to plant could reflect a longer period of time after inoculation of a plant before that plant could itself serve as a source of virus for further spread, or it could reflect a reduced transmission efficiency from plants with resistance to virus replication

Incidence of disease expression, severity of symptoms caused by disease, and timing of disease development can be determined visually in the field. Beyond these observations, the determination of the specific viral function repressed in disease resistance usually involves virus detection technology. It is difficult to distinguish between true immunity to initial infection and failure of virus to move from initially infected cells, since there may be very few initially infected cells, and the virus accumulated in those cells may be insufficient for detection. In either case, infection cannot be demonstrated after repeated inoculation, and the plants may be described as field-immune according to Cooper and Jones ***(4)*** Graft inoculation techniques may be used to distinguish between failure of virus to move from the site of initial infection and its failure to infect new cells after it has moved ***(5)*** Varying degrees of resistance to virus multiplication in plants may be demonstrated using quantitative local lesion assays ***(6)***. Quantitative serological methods may also be used for this purpose, but only to the extent that the differences in virus antigen accumulation detected serologically reflect real differences in virus multiplication Very low levels of virus accumulation in plants below those detectable by clinical methods can usually be demonstrated by graft transmission to susceptible plants ***(5)***, if the virus moves systemically. Tolerance is easily demonstrated by showing that the plant contains virus, but expresses mild or no symptoms

Resistance based on some form of repression of transmission can usually be demonstrated by showing that the plant is susceptible when an alternative means of transmission is used.

### *3.3. Plant Testing*

The objective in field testing is to identify lines that retain or improve the original parental qualities and are also resistant to the virus disease.

#### *3.3.1. Field Screen Tests*

To save time and resources, it is important to eliminate lines that are obviously susceptible, or that have disqualifying type and yield defects, as early as possible in the resistance selection process. A field screen test involving in vitro-propagated plants is recommended for this purpose, since it is easier to handle the large numbers of plants required in a field test than under controlled conditions, and results are more reliable. This test usually involves intentional inoculation with the homologous virus, but natural exposure could be used in instances when exposure is reliable. Major quantitative deviants are often apparent by their lack of vigor during in vitro propagation and in soil pots, and these can be eliminated before they reach the field. Large-scale field replication is not required with artificially inoculated tests (2 replicate plots with 10 plants/plot is usually sufficient), and most decisions to eliminate or retain a line are made by visual observation without the aid of statistical comparison Virus titer and presence or absence of virus among plants of selected lines may be determined as a selection tool, using a range of diagnostic assays

#### *3.3.2. Selection for Agronomic Performance*

Selection for agronomic performance should proceed concurrently with selection for resistance This subject is described in Chapter 51

#### *3.3.3. Virus-Resistance Efficacy Tests*

##### 3.3.3.1 Preliminary Decisions

1 Determine the degree of resistance desired: Practical field immunity (disease is precluded) is a qualitative characteristic that may be illustrated with a single plant. The test for immunity must provide an exposure that eliminates any practical possibility that a susceptible plant would escape infection. Thus, it is more efficient to concentrate on small numbers of plants if this level of resistance is required

A different approach is required when resistance is less than immunity Replication and statistical design are important Furthermore, the severity of virus exposure must be adjusted, so that practical levels of resistance will be tested, but not overwhelmed by excessive inoculation pressure.

Ultimately, resistance is measured as reduced influence of disease on crop yield and quality Factors that may contribute to reduced disease loss include percentage of plants that develop disease, severity of symptoms among diseased plants, length of time required following inoculation for symptoms to develop, concentration of virus in infected tissue, degree of systemic distribution of the virus, capacity of an infected plant to serve as a source of virus for vectors, susceptibility to the spread of virus from plant to plant, numbers of vectors found colonizing plants, frequency of visitation by vectors, and attraction of vectors to virus source plants

2 Determine the kind of resistances that would be effective The specific strategies used for field testing must depend on the kind of resistance desired or required One type of resistance may be effective for one virus, but it might not be effective for another, depending on epidemiological factors involved in dissemination of the different viruses (**Note 3**)

Tolerance generally is not the preferred means of reducing disease loss, but it could be acceptable when the virus is not a threat to other crops (i e., potato virus X and potato virus S of potato), or when infected plants within the crop itself do not serve as a source of virus for infection of other plants (i e , beet curly top virus-infected tomato)

### 3.3.3.2. Resistance to the Homologous Virus

This test involves intentional inoculation of each plant with the virus isolate that served as the source of the transgene. It is used to rank the resistance of lines that survive the initial screen test described above. When incidence of systemic infection is the criterion for resistance selection, at least four replications with 20 plants/rep, arranged in a randomized complete-block design, is recommended

The chief advantage of this approach is that it insures that all plants receive a uniform exposure The exposure is easily limited to the homologous virus for mechanically transmissible viruses, but not for vectored viruses, since it does not exclude natural exposure to other isolates and strains carried to the plots by vectors. Natural exposure to vectors might be limited by conducting the inoculation during a period when the vectors are not present in the area, and then controlling the vector using pesticides or other methods at later stages.

This test provides an opportunity to obtain resistance data on the incidence of systemic infection, time required for disease development after inoculation, virus concentration in infected plants, and severity of symptoms on infected plants. It provides no direct information on resistance to spread of virus from plant to plant. This test also provides an opportunity for more stringent selection of agronomic characteristics among the most resistant lines.

The inoculum pressure should be sufficient to assure high infection rate in wild-type control plants, but should not exceed that required to infect 100% of

wild-type control plants. If it does, there is a risk that the test may not recognize practical levels of resistance that are overwhelmed by excessive inoculum pressure. Plants should normally be inoculated when young, vigorously growing, and susceptible to infection, since older plants may not develop definitive symptoms and may acquire resistance to infection and systemic invasion. The method of inoculation should be based on the means of transmission of the particular virus in nature. Transgenic resistance effective against mechanical transmission has not always been effective against vector transmission *(7)*.

#### 3.3.3.3. Virus Strain Specificity Tests

The purpose of this test is to determine whether a resistance selected for efficacy against the homologous virus is also effective against the major variants and strains of the same virus. This test is similar to the test for resistance to the homologous virus, but it is repeated for each of many different isolates or strains of the virus, and the data taken should be the same. To maintain a manageable size, only a few lines considered for commercialization should be included

Virus strain specificity tests for quantitative characteristics cannot be conducted in the field in areas of high natural disease pressure mediated by mobile vectors. Contamination by natural exposure would compromise the data for any particular isolate or strain. It is a feasible field test if the virus is restricted to mechanical transmission. It is also a feasible test for a vectored virus, provided that the resistance is qualitative and the objective is to determine whether any isolate or strain of the virus, including those that might be introduced to the test by vectors, will break the resistance.

To prevent any possible interference by crossprotection reactions, it is important to preclude or limit infection from outside sources prior to and during the intentional inoculation with specific virus isolates. For this purpose, the plots may be covered with a floating net. Vectors viruliferous with the specific isolate for intentional inoculation are released under the net. The nets may be removed, exposing plots to natural exposure after an appropriate period for inoculation with the specific isolates. If there is a need to minimize exposure after the nets are removed, vector-control measures may be applied throughout the season.

#### 3.3.3.4. Natural Exposure Tests

In natural exposure tests, the experimental plots are planted in a statistical arrangement and exposed to the viruses and vectors that occur in the region at the discretion of nature, with no effort to control the exposure. Ultimately, this is the test that resistance must survive, but there are serious disadvantages to this approach for rapid selection of resistance in the early stages of selection.

Natural exposure may not provide reliable disease exposure, if the disease does not occur in epidemic proportion every year. Although natural exposure is essential in the long run to demonstrate whether strains exist that will overcome the resistance, the range of virus strains that occur in a particular region may be limited. Furthermore, natural exposure in small plots is not reliable for viruses whose epidemiology depends on a few initial infection centers in the field and efficient plant-to-plant dispersal from those infection centers. With such viruses, entire plots may escape exposure if an initial infection is not established in the plot. Natural exposure should involve large plots, should be conducted at multiple locations, should be limited to the few lines in final evaluation for commercialization, and may be associated with yield testing.

A problem in natural exposure trials for a vectored virus is that the susceptible control plots may provide a source of disease pressure to the resistant test lines that would never be encountered in a commercial situation or in a solid stand of the resistant line This exposure may be limited, depending on mobility of the vector, using border rows of immune plants or a barrier screen. If the problem cannot be controlled, it is best to eliminate the susceptible control plots and compare the performance of resistant lines with susceptible control plots that receive commercial vector control practices, or with a standard for susceptible control plants established at a safe distance from the test plots.

Data taken in natural exposure trials should focus on a comprehensive view of the extent to which the resistance is likely to reduce the influence and cost of disease on the commercial crop. Performance of the transgenic lines should be compared with that of the parental line grown under the best of current disease control practices The incidence and severity of virus disease, appearance of resistance-breaking strains, undetected defects, like an unusual susceptibility to a different disease, and ability to match yield and quality standards of the parent cultivar are important characteristics to record.

#### 3.3.3.5 Virus Spread Tests

If most infection within a field is disseminated from relatively few infection centers within the field itself, resistance to spread of virus from one plant to another could provide practical resistance. If the virus depends on mechanical transmission for dissemination, resistance to its spread can be tested in small plots by measuring the rate of virus spread in both directions from a centrally infected plant in a row The influence of various machines and of workers that spread viruses mechanically as they operate in the field is easily measured in this manner. If the virus is vector-dependent, and the vector is relatively mobile and spreads the virus intermittently from one plant to another some distance away, resistance to spread of virus cannot be tested in small plots. However, if

the vector is relatively immobile and spreads the virus from one plant to adjoining plants in a concentric pattern, then resistance to the spread of virus can be tested in small plots, again, by measuring the rate of virus spread in both directions from a centrally infected plant in a row of plants. Infective vectors should be caged on the central plant a few days, until they become acclimatized to the situation and spread virus normally. Alternatively, an infected plant can be transplanted at the center of the row, and virus-free aphids placed on this plant. After an appropriate period of time, to achieve spread of virus from the central plant, the plots are treated with pesticide to eliminate the vectors, and disease reading may be taken after an appropriate incubation period to allow for systemic infection of all infected plants.

### *3.3.4. Risk-Assessment Tests*

A risk associated with the release of plant germplasm expressing viral genes has never been documented. However, there is much speculation ***(8)*** that risks exist, based on potential interactions between transgenes or their products and viruses that infect transgenic plants (further discussed in Chapter 51 and 54) The field is an appropriate place to test for these risks, since the plants in the field are exposed to a much broader range of viruses than could ever be achieved under controlled conditions. Risk assessment observations and tests should be integrated into every field evaluation.

#### 3 3.4.1. Synergism and Complementation

Complementation, the process by which a functional gene of one virus corrects for defectiveness in the same function of another, coinfecting virus, is a well-known phenomenon. Thus, expression of a transgene could induce susceptibility in the transgenic plant to new viruses, if the expressed gene provided an essential function that the new virus could not itself provide. To test this possibility, field-test plots should be examined for specific viruses common in the area, but to which the transgenic species is normally immune.

Synergism is the interaction between an infecting virus and the product of a transgene that results in more severe symptoms than should be expected by infection of the parental cultivar. This occurrence would be indicated by severe symptoms that were not associated with alterations in the infecting virus.

#### 3.3.4.2 Transencapsidation

If a virus infecting a transgenic plant were encapsidated with CP produced by the transgene, the infecting virus could acquire the transmission characteristics and serological properties of the transgene virus. These possibilities are easily tested by checking the transmission properties and serological properties of viruses found infecting transgenic plants.

#### 3.3.4.3. Recombination

A new hybrid virus could be created by genomic recombination between the genome of a virus infecting a transgenic plant and the transgene or its mRNA Sedimentation characteristics of the new hybrid would probably be different from the original virus, and its occurrence might be indicated by production of symptoms unusual for any of the viruses that infect the crop species. More detailed analyses for genomic alterations are recommended when these tests indicate a change has occurred

Alterations in the homologous virus may be difficult to detect, since the alteration may have but minor effects on many properties of the virus Isolating the altered virions would be difficult unless the alteration provided a competitive advantage in the transgenic host or caused a change in host range that would facilitate its isolation.

#### 3 3 4 4 Outcrossing and Weediness

There is a potential risk that a transgene could be introduced into wild species or other crop plants by outcrossing. This risk exists only when plant species that could potentially cross with the transgenic species grow in the region where test plots are located. It may be necessary to determine crossability by experimentation prior to field testing. A number of approaches may be utilized to control this problem. Male sterility in the parental line used for transformation eliminates the possibility of outcrossing. Removal of flowers from plants in transgenic test plots also eliminates the possibility of outcrossing. Isolation of test plots from other plants may also be practical, depending on the distance required to prevent crosspollination. It may also be practical to eliminate species in the area of the test plots that could potentially cross with the transgenic species for the duration of the testing. Screens could be used to exclude pollinating insects

There is a possibility that a crop species that normally will not compete as a weed could acquire this ability by virtue of its transformation with a viral gene. To guard against this possibility, test plot areas should be examined for volunteer plants of the transgenic species for 2 yr following testing. Volunteers must be eradicated.

## 4. Notes

1. A strong root system is achieved in potatoes transplanted to flats from in vitro cultures by watering the plantlets with a 1 250 dilution of a soluble 15-30-15 nutrient solution
2. For potatoes, we find that hand planting hundreds of small plots is faster and neater, and less subject to error than using a transplanter. Potato hills are established and side-dressed with fertilizer using a standard potato planter Holes for

transplanting are punched by hand with a 40-mm-diameter, thin-walled plastic pipe, and 250 mL of 1 125 dilution of the fertilizer described above is applied to the roots of each plantlet soon after it is transplanted.

3. The kind of resistance that will be effective depends on epidemiological factors that control dissemination of the particular virus Dissemination of potato leaf roll virus, for example, depends on relatively immobile apterous aphids that spread virus from few infection sources within the field as they walk slowly from plant to plant Factors such as length of time after inoculation for a plant to become a source of virus and concentration of virus in the source plant could result in resistance to spread of this virus. Beet curly top virus in tomatoes, on the other hand, rarely if ever spreads from tomato to another plant. Tomato is poisonous to the vector Although this stimulates the highly mobile vector of beet curly top to hyperactive movement and efficient transmission initially, it reduces acquisition of the virus and causes the vector to stop feeding within 4 h, and to die later *(9)*

## References


1 Kaniewski, W K and Thomas, P E (1993) Field testing of virus resistant transgenic plants *Semin Virol* **4,** 389–396

2 Allard, R W (1960) *Principles of Plant Breeding* Wiley, New York

3 Toyoda, H , Chatani, K , Matsuda, Y , and Ochi, S (1989) Multiplication of tobacco mosaic virus in tobacco callus tissues and in vitro selection for viral disease resistance *Plant Cell Reports* **8,** 433–436.

4 Cooper, J I. and Jones, A T (1983) Responses of plants to viruses proposals for the use of terms. *Phytopathology* **73,** 127,128

5. Hassan, S and Thomas, P. E (1988) Extreme resistance to tomato yellow top virus and potato leaf roll virus in Lycopersicon peruvianum and some of its tomato hybrids. *Phytopathology* **78,** 1164–1167

6. Holmes, F O. (1929) Local lesions in tobacco mosaic *Bot Gaz* **87,** 39–55

7. Ploeg, A. T , Mathis, A , Bol, J. F., Brown, D J. F., and Robinson, D J (1993) Susceptibility of transgenic tobacco plants expressing tobacco ratle virus coat protein to nematode-transmitted and mechanically inoculated tobacco rattle virus *J Gen Virol* **74,** 2709–2715

8. de Zoeten, G A (1991) Risk assessment do we let history repeat itself? *Phytopathology* **81,** 585,586

9 Thomas, P E (1972) Mode of expression of host preference by *Circulifer tenellus*, the vector of curly top virus *J Econ Entomol* **65,** 119–123

# 51

# Agronomic Performance of Transgenic Plants

**Peter E. Thomas and Wojciech K. Kaniewski**

## 1. Introduction

This chapter describes methods to assess agronomic performance among plants of existing plant cultivars that have been transformed with viral genes for the purpose of achieving resistance to the virus *(1)*. The principles and general guidelines for selection among plants altered by transformation are the same as those established for classical breeding *(2)*. However, the approach may be somewhat different, since the objective of the transformation is to alter only one characteristic of the cultivar, its virus disease resistance, while retaining the agronomic characteristics of the parental cultivar. This can be the objective of classical breeding, but breeding tends to affect a much broader range of characteristics and to create entirely new cultivars. The chief advantage of achieving virus resistance by the transgenic approach, as compared to breeding, is that resistance may be added to an established cultivar with little or no alteration in the proven agronomic performance of the cultivar. There may be considerable market resistance to the introduction of a new cultivar, since culture, storage, and processing must be adapted to the requirements of the new cultivar, and because market appeal has not been established for the new cultivar. Because of this resistance to new agronomic characteristics, the objective in development of transgenic virus resistance is to duplicate the parental line in all aspects except virus disease susceptibility. Fortuitous improvements in agronomic characteristics that may result from transformation may not be acceptable, depending on market requirements.

Agronomic assessment and selection among plants transformed for virus resistance is required, because transformation with viral genes may change plant characteristics that are agronomically important. Two types of changes occur. One is somoclonal variability *(3)*, caused from a restructuring and

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

realignment of the genetic code as new genes are inserted. Such changes are common and may be subtle among transformed plants. Another type of change is caused by the direct expression of the inserted gene *(4)*. Rigorous selection among hundreds of transformants may be required to identify lines that are virus-resistant and that also conform to or exceed standard characteristics of the parent cultivar Selection for resistance and agronomic performance should proceed concurrently

In contrast to resistance, agronomic performance involves many characteristics, including such qualities as type, yield, foliage appearance, time to maturity, surface appearance, color, smell, taste, texture, shape, size, cooking and processing quality, bruisibility, storability, and shipping quality. The relative importance among these characteristics depends on the crop and the use for which it is intended. Some agronomic qualities are objective and qualitative, are easily assessed in small tests, and often conform closely with that of the parent cultivar *(5)*. Others, particularly yield, are quantitative, and some, like taste, are both quantitative and subjective. These may require extensive testing for accurate assessment.

## 2. Materials

Agronomic performance is markedly affected by subtle differences in growing conditions and must be tested under field conditions. Thus, the first and most important requirement for the assessment of agronomic performance is a satisfactory field site. It should present conditions representative of those under which the crop is grown commercially, but this presents a problem, because the virus disease is a production problem in the area for which the new resistant line is intended, and presence of the disease in the susceptible parental line may prevent a valid comparison between the transgenic and parental lines. The best solution is to locate agronomic performance trials in the production area and provide the healthy parental control by using pesticides to prevent infection. When that is not possible, it may be necessary to locate the trials in an area where the disease is not a problem and accept the fact that performance may not be the same there as in the production area. Performance in agronomic trials may be ignored among all lines that fail in the resistance trials.

Culture, cultivation, and harvest machinery are required for field testing. Standard machinery developed for small farms and market gardeners is often adequate or easily modified for small-plot work. Planting and harvest machinery capable of dealing with many small plots without mixing between plots or even between individual plants is often extremely desirable, but very difficult to acquire, adapt, or construct. It is frequently faster, neater, and more reliable to plant and harvest by hand than to use machinery designed for commercial

production, especially in early stages of selection, when many lines are under test in many small, replicated plots. Cultural management of small test plots is a real test of adaptability and resourcefulness of the manager.

A computer database is required to manage data. Appropriate statistical software is needed to assist in designing plot size and replication, and to statistically analyze differences in final results.

A tissue-culture laboratory and growth chamber/greenhouse/screenhouse facilities are usually required if in vitro-produced plantlets are used for field testing. Controlled plant growth facilities may be required to propagate plants to be tested before moving them to the field. A fully equipped analytical and molecular laboratory is necessary to determine chemical and biochemical composition of transgenic plants

## 3. Methods

### *3.1. Plant Culture*

#### *3.1.1. Field Preparation*

Cultivation, of pre-emergence herbicide for weed control, and treatments to control soil pathogens should be applied. In general, the standard treatments used in commerce for the parental cultivar are desirable, since they provide the conditions under which the transgenic cultivar must perform.

#### *3.1.2. Propagation of Transgenic and Control Lines*

The type of propagants used in field testing will depend largely on whether the crop is grown from true seed or vegetative parts.

##### 3 1.2.1. Vegetatively Propagated Plants

Since in vitro plantlets are immediately available following transformation, it may save time to proceed with initial testing of lines using in vitro-produced, $R_0$ plantlets Although vegetatively produced crops are always grown in the $R_0$ generation, the size and physiological condition of plant parts used as seed can markedly influence yield and quality. Thus, final field evaluation should not utilize in vitro plantlets as the seed, but should utilize the seed of commerce. However, it is often difficult to produce vegetative seed free of contamination with seed-borne viruses or other pathogens that may interfere with evaluation. In such cases, in vitro plantlets may be maintained as a backup.

##### 3.1.2.2. Seed-Grown Plants

In crops that are produced from true seed, it is much easier and less labor-intensive to use true seed than vegetatively propagated plantlets, even in initial field testing If the transgenic species is genetically self-compatible, $R_1$ segre-

gating progeny can be used in initial screening. This will save time, but it must be understood that, in lines with a single gene insertion, 25% of progeny will not contain the transgene, 50% will be heterozygous for the gene (contain only a single copy), and 25% will be homozygous for the gene Heterozygous plants may not be as resistant as homozygous plants among segregating progeny. If the species requires outcrossing for seed production, there may be a distinct advantage to eliminating susceptible and off-type lines at the vegetative stage, prior to selecting parents for homozygous seed production This is particularly important if resistant phenotypes are relatively rare among the lines.

### 3.1.3. Growing and Transplanting In Vitro-Produced Plantlets

In vitro-produced plantlets normally cannot be transplanted directly in the field They first must be transferred to sterile soil or artificial planting medium in small pots and then transplanted to the field after a period of growth and soil adaptation. It is convenient to use plastic or styrofoam flats for this purpose. The young plantlets in flats should be shaded from direct sunlight in the first few days after transplanting. Sanitation is very important. Flats should be placed on a pathogen-free surface, since *Pythium* and other damping-off fungi can invade the sterile planting media and kill the young plantlets rapidly, if the flats are placed on a contaminated surface. This can result in a complete loss of the planting A balanced formulation of major and minor elements must be added, especially when artificial planting media are used, and the amount applied should increase as the plantlets grow and require more nutrients Development of a strong root system is especially important, not only for a fast start once the plantlets are transplanted to the field, but also to prevent the planting medium from falling away from the roots as the plantlets are lifted from their pots and transplanted to the field (**Note 1**)

Plantlets should be transplanted from the flats to the field while they are growing vigorously Transplanting machinery may be used for this purpose, but the process requires meticulous organization to prevent errors, when hundreds of small, replicated plots are involved (**Note 2**)

### 3.1.4. Plant Care During Growing Season

Most cultural practices, including irrigation, fertilization, and weed control, should be those used in commercial production of the crop

### 3.1.5. Harvest and Storage

Small-plot harvesting without mixing between plots and without labeling mistakes is essential, but difficult to achieve. Careful planning and close supervision is essential. Appropriate storage is especially important in cases in which

the virus disease affects storability of the crop. It is difficult to achieve when refrigeration, humidity, and control of atmospheric gasses are required.

### *3.2. Plant Testing*

Selection for agronomic performance among transgenic lines is largely a process of eliminating lines that do not meet performance standards of the parental cultivar (**Note 3**). It begins with elimination of lines with obvious defects and progressively becomes more stringent, until final selections require large-scale testing with careful statistical analysis. The selection process becomes progressively more costly at each step, and much saving can be realized if defective lines are identified and eliminated early in the process.

#### *3.2.1. Agronomic Appearance of New Transformants*

Off-type plants can often be identified among new transformants in tissue-culture media by their lack of vigor or by conformational aberrations. Transformants with distinct and serious defects may be eliminated immediately. However, a degree of caution must be exercised in discarding the initial transformants. These plants sometimes exhibit some transitory off-type growth that may be caused by stresses associated with regeneration or with the tissue-culture medium.

#### *3.2.2. Agronomic Appearance of Tissue-Culture-Propagated Plants*

The next step in the selection process after regeneration of transformed plants involves micropropagation of the transformants in vitro and transfer to soil flats Off-type lines, especially those with low vigor, are more easily recognized when they are viewed as groups of plants in flats and compared with control plants propagated at the same time than when viewed as individual plants. In our experience, transformed potato plants that grow slowly in soil flats always perform poorly when they are transferred to the field. Aberrant growth in soil flats is sometimes a transitory condition.

#### *3.2.3. Agronomic Characteristics of Plants in First-Year Field Tests*

More subtle vigor and growth aberration defects that are not obvious at an earlier stage are often exhibited after plantlets are transferred from soil flats to the field, and most lines that do not rate serious consideration for commercialization can be eliminated in the first year of field testing, either on the basis of susceptibility to virus disease or poor agronomic performance. It is important to remember that the micropropagated plantlets used in many early trials of some crop species may not produce the same agronomic performance as the seed used in commerce. Agronomic performance is important only among lines that exhibit adequate disease resistance. Vigor and foliage characteristics of

transgenic lines may be rated relative to that of the parental cultivar in disease-elimination trials only if this rating can be conducted prior to the development of disease in the susceptible parent or when disease in the susceptible parent can be controlled. For the purpose of rating vigor, it is convenient to use a visual rating scale of 1–5, with the parental cultivar automatically assigned a rating of 3.

It is important to remember that plant vigor, growth aberration, and disease resistance are important only as these characteristics are reflected in yield and agronomic quality of the crop. Therefore, early season consideration of these factors is important in field tests only to the extent that they may direct harvesting and further evaluation efforts to the best lines at the end of the season. In our experience with potatoes, we have always rated the yields of all lines at least visually at harvest, and we weigh the yields only of the best lines in first year tests. Lines with poor vigor have always produced poor yields, but lines with good vigor have not always produced high yields or tubers with appropriate size and conformation Although growth aberration is usually associated with poor vigor, it does sometimes occur in vigorous lines, but such lines have usually produced low yield and off-type tubers

### 3.2 4. Agronomic Properties of Selected Lines by Multiyear and Multilocation Tests

Agronomic performance is markedly affected by a multitude of environmental and management practices Potatoes, for example, are affected by water and fertility, soil texture and microflora, daylength and temperature, and the requirements for one cultivar may differ from those of another. Thus, testing in different regions where the crop is produced is necessary to ensure that lines selected in one region retain both their resistance and agronomic performance when grown in other regions where epidemiological and environmental conditions may differ Testing can proceed in different regions simultaneously to save time, but it may be more cost-effective to select lines, first in one region, and then test whether the resistance and agronomic characteristics are expressed in the variety of regions where the crop will be produced.

The agronomic characteristics of transgenic lines may differ somewhat from the parental line, and the characteristics of lines intended for commercialization should be defined in detail. The new lines should be described following the guidelines provided by the International Union for the Protection of New Varieties of Plants (UPOV) ***(6)***. UPOV provides for protection of breeders' rights, based on criteria that include distinctness, homogeneity, and stability of a variety, and has published guidelines for the conduct of tests to determine these criteria. These guidelines are adapted for the special requirements of each genus and species and provide information on the detailed information needed

on varietal characteristics. By following these guidelines, member states are provided a common basis for testing varieties and establishing variety descriptions in a standardized form that facilitates international cooperation in examination between their authorities.

### *3.2.5. Yield Assessment Tests*

Quantitative characteristics, particularly yield, require large-scale field testing over several years and in many locations. Only lines considered for commercialization should be included. The design of yield tests must account for the many subtle cultural factors that affect yield, such as relative foliage vigor of plants in the next row, planting date, physiological condition and size of plant parts used as seed in vegetatively propagated crops, response to fertility, water management, and conditions, and time from planting to maturity. Experimental design and data collection and processing require careful statistical treatment *(7)*. A convenient formula *(8)* to determine the number of replications needed to determine differences in yield at the 95% confidentiality level is as follows.

$$\mathrm{LSD} = 2^{1.5} \times CV \times N^{-0.5}$$

where LSD = least significant difference, $CV$ = variation coefficient (usually about 10%), and $N$ = number of replications.

### *3.2.6. Agronomic Performance Under Virus Pressure*

Ultimately, the transgenic line must perform well under the virus inoculation pressure of the production area for which it is intended. The level of resistance and of agronomic performance required to replace the parental cultivar will be determined by many factors, including the degree to which the disease can be controlled in the parental cultivar, the cost of that control, and environmental and food safety risks associated with applying the control.

### *3.2.7. Gene Identity, Localization, Integrity, and Stability*

Prior to commercialization of virus resistant transgenic plants, it is necessary to know as much as possible about the gene that has been incorporated into the plant Some of this information is required by regulatory agencies, and specific regulatory requirements vary from country to country. Additional information will serve to expand our information about the properties of newly generated transgenic plants. This information is of special interest to breeders and researchers assessing the risks associated with introducing transgenic plants into agriculture. Complete knowledge of the molecular composition of transgenic plants generated will also facilitate their identification for property rights.

The complete sequence of the resistance gene used, as well as sequences of all other genetic elements used, should be known prior to integration into the plasmid. On occasion, there are abnormalities associated with *Agrobacterium*-mediated transformation, and abnormalities may be much more common in plants produced via particle-gun transformation.

Initially, new transformants are assayed by Southern blotting to detect inserted DNA and to determine if the coding sequence is intact (correct size of the DNA) (Chapters 42 and 43). Northern assays confirm the presence of RNA, and, therefore, transcription (Chapter 44). Finally, the presence of, and correct size of, the translated protein is determined by Western blot analysis (Chapter 45) (**Note 4**).

In plants grown from seed, it is usually suitable to continue only with plants containing a single insert of the gene of interest. Proper segregation (3:1) of $R_1$ progeny indicates the presence of one insert. This and the number of copies in the insert can be confirmed molecularly by Southern blotting. DNA analysis (Southern blotting and PCR) allows detection of fragmented gene inserts or backbone sequences from the plasmid (particle gun transformation) or leaking plasmid left border sequences (*Agrobacterium*-mediated transformation) (Chapters 41–43). For breeding purposes, it may be important to determine in which chromosome and where the gene is inserted by various mapping techniques.

Gene stability through successive plant generations is a major concern. Stable expression and performance of the gene need to be confirmed in at least six generations to accept the line for practical use and breeding.

### *3.2.8. Nutritional Composition, Chemical Analysis, and Quality Assessment of the Final Product*

Food safety of genetically modified foods is a critical issue in development of transgenic plants. Governments around the world regulate the commercialization of genetically modified crops and require a wide range of analyses to ensure their food and environmental safety. The World Health Organization (WHO) is playing a leading role in developing internationally accepted principles and procedures for the evaluation of safety of foods produced by biotechnology. Important principles concluded by the WHO in a recent international workshop *(9)* include the following: A new food or food component found to be substantially equivalent to an existing food or food component should be treated in the same manner as the existing food with respect to safety; where substantial equivalence could be established for a new food in all aspects except for the inserted gene or its product, the safety assessment should focus on the latter, and a determination that a new food was not substantially equivalent to an existing food does not mean that the new food is unsafe, but

rather that it should be evaluated on the basis of its own unique composition. The WHO report ***(9)*** provides guidelines for determining substantial equivalence and provides case studies that may serve as examples of the kinds of analyses needed to establish the safety of food products from transgenic plants.

Safety assessment to gain regulatory approval for commercialization of potatoes with transgenic resistance to the Colorado potato beetle ***(10)*** concentrated on demonstrating that newly introduced proteins not found in the parental cultivar were safe and that transgenic tubers were substantially equivalent to the parental cultivar with regard to 23 other key components, including total solids, sugars (dextrose and sucrose), proximate composition (total protein, fat, carbohydrate, total dietary fiber, calories, and ash), vitamins (vitamin C, thiamin [vitamin B1], pyridoxine [vitamin B6], folic acid, niacin [vitamin B3], riboflavin [vitamin B2]), minerals (calcium, copper, iodine, iron, magnesium, phosphorus, sodium, potassium, and zinc), and natural glycoalkaloid toxicants (solanins and chaconines).

Nutritional analyses are needed to satisfy requirements for nutritional information that must be presented in the labeling (the label is called "Nutrition Facts" in the United States) on foods marketed in some nations of the world. These analyses are a part of the quality assessment aspect of agronomic performance, which may be reserved until final line selections are made for commercialization.

Other qualities assessed in the final stages of selection will depend on the crop and the importance of the quality. Additional qualities we assess in the final stages of transgenic potato development include incidence of hollow heart, brown center, internal brown spot, potato leaf roll virus-induced net necrosis, and other internal tuber defects, susceptibility to blackspot bruise, French fry color for tubers stored at 4.5 and at 7.3°C, incidence of sugar end, and severity of sugar end.

## 4. Notes

1. A strong root system is achieved in potatoes transplanted to flats from in vitro cultures by watering the plantlets with a 1.250 dilution of a soluble 15-30-15 nutrient solution
2. For potatoes, we find that hand planting hundreds of small plots is faster and neater, and less subject to error than using a transplanter. Potato hills are established and side-dressed with fertilizer, using a standard potato planter. Holes for transplanting are punched by hand with a 40-mm diameter, thin-walled plastic pipe, and 250 mL of 1:125 dilution of the fertilizer described above is applied to the roots of each plantlet soon after it is transplanted.
3. In practice, the stringency imposed on type and yield conformity will be determined arbitrarily and will depend on many economic factors Certainly, a loss equivalent to the cost of current disease control measures, plus the cost of current disease losses, should be tolerated.

4 In most cases, it is possible and convenient to analyze gene expression products by ELISA *(11)*. It is usually used for determining expression in segregating $R_1$ progeny Saving the highest $R_1$ expressors for seeds greatly increases the chances for selecting plants with two gene copies, which will produce homozygous lines Confirmation of homozygosity is also done via ELISA, usually by analyzing expression of about 30 $R_2$ progeny plants. In case of difficulties in reliably detecting virus-resistance genes, it is convenient to locate transformed plants by assaying for the presence of selectable marker proteins (i.e , NPTII) or scorable proteins (i e , GUS) by ELISA

## References


1. Kaniewski, W. K and Thomas, P E (1993) Field testing of virus resistant transgenic plants *Semin Virol* **4,** 389–396
2 Allard, R W (1960) *Principles of Plant Breeding* Wiley, New York
3 Toyoda, H., Chatani, K , Matsuda, Y., and Ochi, S (1989) Multiplication of tobacco mosaic virus in tobacco callus tissues and in vitro selection for viral disease resistance *Plant Cell Rep* **8,** 433–436
4 Lodge, K., Kaniewski, W., and Tumer, N (1993) Broad spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein *Proc Natl Acad Sci USA* **90,** 7089–7093
5 Jongedijk, D., Huisman, M J , and Cornelissen, B J C (1993) Agronomic performance and field resistance of genetically modified, virus-resistant potato plants *Semin Virol* **4,** 407–416
6. International Union for the Protection of New Varieties of Plants (UPOV) (1979) Revised general instruction to the guidelines for the conduct of tests for distinctness, homogeneity, and stability of new varieties of plants
7. Mead, R., Curnow, R N , and Hasted, A M (1993) *Statistical Methods in Agriculture and Experimental Biology* Chapman and Hall, London
8. Little, T M and Hills, J F (1978) *Agricultural Experimentation Design and Analysis* Wiley, New York
9. World Health Organization, Food Safety Unit (1995) Application of the principles of substantial equivalence to the safety evaluation of foods or food components from plants derived by modern biotechnology. Report of a WHO workshop (WHO/FNU/FOS/95 1) held in Copenhagen, Denmark, 31 October–4 November, 1994
10 Lavrik, P. B , Bartnicki, D E., Feldman, J., Hammond, B G., Keck, P J , Love, S L , Naylor, M. W , Rogan, G J , Sims, S. R , and Fuchs, R. L (1995) Safety assessment of potatoes resistant to Colorado potato beetle, in *Genetically Modified Foods* (Engel, K K., Takeoka, G R , and Teranishi, R , eds ), ACS Symposium Series 605, American Chemical Society, Washington, DC, pp. 148–158
11 Clark, M F and Adams, A N (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses *J Gen Virol.* **34,** 475–483.

# 52

# Mechanisms of Resistance

## *Expression of Coat Protein*

**Ulrich Reimann-Philipp**

## 1. Introduction

The expression of viral coat protein (CP) genes in transgenic plants can lead to different phenotypes of resistance *(1)*. Occasionally, transgenic plants escape infection completely and do not accumulate virus or develop symptoms In other cases, local and systemic virus accumulation and development of systemic infection proceed at a rate slower than in nontransgenic plants. In transgenic plant lines, the proportion of plants that develop symptoms after inoculation is frequently lower than in control lines. It has also been shown that transgenic plants can become locally infected and accumulate virus in the inoculated leaf, but do not support systemic infection. The different phenotypes of resistance suggest that there is not one common mechanism by which virus infection is affected in transgenic plants, but different steps of virus infection are inhibited in different host–virus combinations.

When coat protein-mediated resistance (CPMR) or coat protein-mediated protection (CPMP) of tobacco to tobacco mosaic virus (TMV) was first demonstrated, the degree of resistance correlated with the level of CP accumulation in the transgenic plants *(2)*. Those plant lines that accumulated higher levels of CP were more resistant to TMV than those with low CP accumulation. Since then, it has been attempted to apply the approach to a variety of other host–virus combinations, in many cases successfully. In some examples, the degree of resistance was also correlated with CP accumulation, in others, that was not the case. Plant lines with low CP levels, or even with no detectable CP accumulation, were shown to be highly resistant. This indicated that CP gene products can lead to virus resistance in different ways, and that in some cases the tran-

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

scripts of the transgene, rather than the protein itself, were effective. Because high protein levels are usually associated with high transcript levels, correlation of CP levels with the degree of resistance suggests, but does not prove, that CP, rather than RNA, is effective. In some cases, the CP levels decline after subjecting the transgenic plants to elevated culture temperatures, the transcript levels change only little or not at all. If CPMR breaks down at high temperatures and is restored after shifting back to normal temperature, a protein effect is likely. The only sure way to exclude a protein effect is to transform plants with a gene construct that encodes a nontranslatable CP gene, usually a frame-shift mutation, and test the resulting plants for resistance The characteristics of CPMR have not been studied in detail in all the host–virus combinations to which the approach has been successfully applied. In this chapter, some examples will be used to describe possible ways of interference of CP that accumulates in transgenic plants with virus infection. Mechanisms of resistance in cases in which protection is probably caused by RNA will be described in the following chapter.

## 2. The Viral Infection Cycle

Although there is a great variety in genome structure, replication strategies, and manners of transmission between plant viruses, most plant virus infections proceed in similar phases. **Figure 1** shows a model of the infection process and the steps that might be affected in transgenic CP-accumulating plants The initial event is the introduction of one or more virus particles into single cells, either mechanically through wounding or by a vector organism. Inside these cells, the virions disassemble to release the viral genome, which, in most cases, is composed of one or more single-stranded RNA molecules. The viral genes are then transcribed either directly from the genomic RNA or from subgenomic RNAs that are synthesized using complementary replication intermediates as templates. Viral gene products are involved in genome replication, virion formation, and passage of infectious units to adjacent cells, where a new round of replication occurs. Plants that carry hypersensitivity genes respond to local infection with the formation of necrotic lesions, resulting in the arrest of virus spread, and induction of systemic defense mechanisms that prevent or delay subsequent infections. In plants that are susceptible, the infection spreads through the plasmodesmata from cell to cell, until it reaches the vascular tissue and infectious units can enter the phloem. Rapid systemic spread seems to occur mainly through the sieve elements with the assimilate flow. Little is known so far about the mechanisms of entry into, and exit from, the sieve elements.

Plant viruses are transmitted between plants in different ways, most commonly by mechanical means or through insect vectors. Insects usually acquire virus from systemically infected tissue through chewing or directly from the

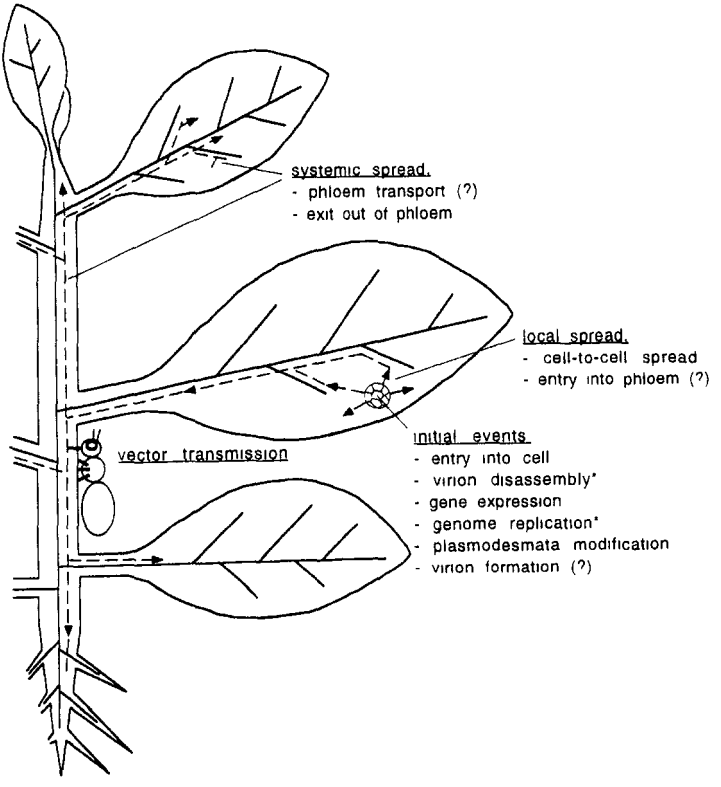



Fig 1 Model of a plant virus infection The model shows different events in virus infection Dashed lines indicate virus spread. Listed are events that could possibly be affected by accumulation of functional or nonfunctional CP in transgenic plants *, evidence for interference has been found. ?, interference is likely, but has not yet been clearly shown

phloem through sucking. Mechanical transmission can occur through direct contact of infected and noninfected plants or through tools or handling.

CPs of some plant viruses function not only in determination of the virion structure and protection of the viral genome. For example, alfalfa mosaic virus (AlMV) replication can only occur in the presence of CP *(3)*. The CP molecules have a high affinity for viral RNA and bind to the genomic RNAs. It is also thought to be involved in regulating the balance between the synthesis of positive and negative RNA strands during replication *(4)*. In viruses that move from cell-to-cell in the form of virions, CP is involved in local spread. Other viruses, such as TMV, can spread locally without CP, but require it for long distance transport through the phloem. In some cases, CP has been shown to

determine the specificity of virus transmission through insect or fungus vectors *(5–9)*.

Functional or nonfunctional CP that accumulates in transgenic plants can interfere directly with one or more steps in virus infection, depending on the function(s) of CP in the particular plant–virus interaction. It is also possible that the presence of CP elicits plant defense responses that result in enhanced resistance to pathogen infection. So far, there is only little evidence for this role in CPMR.

## 3. Tobacco Mosaic Virus

The extensive knowledge of virion structure, genome organization, replication, and spread of TMV has facilitated studies about the mechanism of CPMR in tobacco. Transgenic tobacco plants that accumulate TMV CP either escape infection or develop symptoms slower than nontransgenic controls *(2)* In cultivars that carry the N hypersensitivity gene from *Nicotiana glutinosa*, few or no necrotic local lesions are formed after inoculation *(10)*. There is a clear correlation of CP accumulation with resistance: The plant lines that accumulate the highest levels of CP are most resistant. At elevated temperature, CP, but not the transcript levels, decline in transgenic plants, and resistance breaks down *(11)*. Furthermore, transgenic plants expressing a nontranslatable CP gene are not resistant *(12)*. These findings suggest that the CP itself interferes with TMV infection.

Accumulation of the pathogenesis-related protein PR 1a was determined in transgenic plants that did or did not bear the N-gene, in order to investigate whether plant defense responses are involved in the resistance phenotype *(13)*. Only transgenic plants carrying the N gene accumulated elevated levels of the pathogenesis-related protein PR 1a in the absence of infection; CP accumulation led to TMV resistance in all transgenic plants, indicating that CP probably interfered directly with virus infection, and that plant defense responses do not significantly contribute to resistance.

CPMR of transgenic plants accumulating TMV CP is restricted to TMV and related tobamoviruses. The degree of resistance correlates with the degree of homology between the CP amino acid sequences. There is little or no resistance to viruses from other groups *(14)*. Specificity of CPMR to the virus from which the CP gene is derived, and to closely related viruses, has been generally observed in different plant–virus combinations. In contrast, systemic resistance that develops after hypersensitive response is broad and effective against pathogens that are not related to the inducing one.

There are several indications that early events in TMV infection are inhibited in transgenic tobacco plants. CPMR is largely overcome when TMV genomic RNA or partially uncoated TMV is used as inoculum, instead of TMV

virions. There is only a slight delay in the development of systemic symptoms, compared to nontransgenic control plants. Protoplasts prepared from transgenic plant tissue do not support TMV replication after electroporation with TMV virions, but do so when electroporated with TMV RNA or partially uncoated TMV ***(15)***. Transgenic plants have been generated that express the TMV CP gene from different tissue-specific promoters. These plants accumulated CP either mainly in the upper leaf epidermis, the mesophyll, or the phloem. Only plants that accumulated CP in the upper epidermis, the site of initial infection under laboratory conditions, showed resistance ***(16)***.

Apparently a step prior to virion disassembly is blocked in transgenic tobacco cells. This could be the initial dissociation of some CP molecules from the 5' end of the viral RNA, a process called swelling. Virion disassembly is then completed by binding of ribosomes to the exposed RNA and translation of the TMV replicase proteins from the genomic RNA. It is not yet known whether swelling is caused by the chemical environment in the cytoplasm alone, or whether it occurs at specific sites inside the cell that function as receptors that recognize the 5' end of TMV rods. The latter was suggested by studies in which protoplasts were coelectroporated with isolated CP and TMV ***(17)***. TMV CP can aggregate to different states, depending on the salt and pH conditions. Large aggregates that resembled virions were more efficient in inhibiting TMV replication than small aggregates or monomers, suggesting that the larger CP aggregates blocked an intracellular swelling site. However, it was not possible to monitor the aggregation state of the CP in the protoplasts after electroporation, and the different efficiency of inhibition might have been caused by differences in uptake or stability of the CP Furthermore, in vitro-generated TMV mutants with altered virion surface structure were unable to overcome resistance of plants accumulating wild-type TMV CP ***(18)***. It is possible that an equilibrium exists between release of CP molecules from the 5' end of the TMV RNA and binding of CP that accumulated in the transgenic cells, resulting in stabilization of the virion.

Inoculation with TMV RNA does not completely overcome CPMR. Local and systemic spread of TMV after inoculation with TMV RNA is delayed in transgenic plants ***(19)***. It was also observed that movement of TMV through transgenic stem sections that were grafted into nontransgenic tobacco plants was delayed, but only if the transgenic section contained a leaf that might have functioned as a sink for infectious units. TMV accumulation in protoplasts prepared from transgenic plants occurs slower than in protoplasts prepared from control plants after electroporation with TMV RNA, except when very high levels of TMV RNA were used. These results suggest that TMV replication in transgenic cells is not only affected at the step of virion disassembly, but also at a later stage. There is no convincing evidence yet for interference with cell-to-

cell transport through the plasmodesmata or with systemic transport through the sieve elements.

## 4. Alfalfa Mosaic Virus and Tobacco Streak Virus

The requirement for CP accumulation for CPMR in tobacco to AlMV was demonstrated using transgenic plants that expressed the AlMV CP gene into which a frameshift mutation was introduced *(20)*. The resulting transgenic plants accumulated transcripts, but not CP, and were not resistant to AlMV. Expression of the wild-type CP gene led to protein accumulation and resistance. Transgenic tobacco plants that carried the N gene did not form local lesions after inoculation with the necrotizing YSMV strain of AlMV When progeny of transgenic tobacco plants that did not carry the N gene were infected with AlMV, only a low percentage of plants developed symptoms. Resistant plants accumulated no AlMV in inoculated or systemic leaves, indicating that CP accumulation interfered with an early stage of infection. It is not known whether virion disassembly is affected, because both susceptibility and resistance to infection by isolated AlMV RNAs has been reported *(21–24)* The different results might be a result of different CP levels in the transgenic plants or the different AlMV strains that were used.

AlMV particles contain four different RNAs, of which RNA-4 encodes the CP. To be infectious, an RNA inoculum must contain all four RNAs, or CP must be added. The CP binds to all four RNAs and is required for replication to occur *(3,4)*. Mixtures of RNAs-1–3 have been shown to be infectious on transgenic plants, indicating that the accumulated CP is functional *(24)*. Transgenic plants that accumulated the CP from tobacco streak virus (TSV), a member of the closely related ilarvirus family, could also be infected with AlMV RNAs 1–3 *(20)*. The same plants were highly resistant to TSV, but not to AlMV It was also shown that transgenic tobacco plants expressing a mutant AlMV CP gene encoding a single amino acid change close to the N terminus were susceptible to AlMV infection *(21,22)*. However, the mutant CP was still able to bind AlMV RNA-4 in gel-shift assays. Apparently, different parts of the CP function in CPMR and in replication.

Transgenic tomato plants expressing the AlMV CP gene have also been shown to be resistant to AlMV infection *(22)*. After inoculation, no necrosis developed on inoculated leaves and very little AlMV accumulated. No AlMV could be detected in systemic leaves of resistant plants, indicating that an early step in virus infection was affected.

## 5. Potato Virus X

Potato virus X (PVX) is a member of the potexvirus group and a major pathogen of potato. The CP gene of PVX has been expressed in transgenic

tobacco *(25)* and potato plants *(26)*. In tobacco, the CP levels correlated with the level of protection against PVX infection. The majority of transgenic plants did not develop systemic symptoms after inoculation with up to 5 μg/mL PVX, and accumulated very low levels of PVX in inoculated and systemic leaves. There were significantly fewer local infection sites on inoculated leaves of transgenic plants, compared to control plants. The results suggest that an early event in PVX infection was affected in the transgenic plants. However, in contrast to the TMV–tobacco interaction, protection was not overcome by inoculation with PVX RNA, indicating that virion disassembly was not the major step that was inhibited. The reduction in the number of local infection sites and in virus accumulation in inoculated leaves was also observed in RNA inoculated plants. There was no evidence for interference with long-distance spread of PVX. It is possible that an RNA effect contributed to protection, because expression of an antisense CP gene transcript also led to protection, but was far less effective than the positive-sense gene.

Transgenic potato plants that expressed the PVX CP gene alone or together with the CP gene from the potyvirus potato virus Y (PVY) accumulated CP and escaped infection by PVX after inoculation with up to 5 μg/mL PVX *(26)*. There was no detectable accumulation of virus in inoculated transgenic plants and no tuber transmission. The absence of even a low level of virus accumulation indicated that an initial event in PVX infection was inhibited Since the study did not include RNA inoculations, it cannot be concluded whether this step was virion disassembly. The protection was virus-specific: There was no protection against PVY. However, double transgenic plants that accumulated both PVX and PVY CPs were protected against both viruses when inoculated separately or simultaneously. Transgenic plants expressing the PVY CP gene accumulated transcript levels that were comparable to those of plants expressing the PVX CP gene, but less CP. Studies on tobacco etch virus and other potyviruses suggest that CPMR against potyviruses is caused by an RNA effect rather than a protein effect (**ref.** *27*; further discussed in Chapter 53).

## 6. Cucumber Mosaic Virus

Transgenic tobacco plants expressing the CP gene from the cucumovirus cucumber mosaic virus (CMV) do not develop systemic symptoms after inoculation with CMV virions or isolated RNA. However, virus accumulation in inoculated leaves could occasionally be observed *(28)*. Protoplasts isolated from transgenic plants support CMV replication after electroporation with RNA, but not with virions. Apparently, both initial infection at the stage of virion disassembly and long-distance spread are inhibited in the transgenic plants

A host-specific role in long-distance spread of CMV has recently been shown for the infection of cucumbers *(29)*. Tomato aspermy cucumovirus (TAV) can

infect cucumber cells and spread locally in inoculated leaves, but is unable to cause systemic infection. Addition of CMV RNA-3, but not the other CMV RNAs, to the inoculum complemented for the defect and led to systemic infection by TAV. Complementation was also observed when the RNA-3 carried a mutation that left only the CP gene intact. Different properties of the plasmodesmata that connect mesophyll cells and mesophyll to phloem-parenchyma cells have been shown by dye-injection studies using transgenic tobacco plants that express the TMV movement protein gene *(30)*. It is possible that the CMV CP is involved in the transport of infectious units from mesophyll cells into phloem cells and that accumulation of CP in transgenic plants interferes with that process.

## 7. Common Features of CPMR

Accumulation of viral CP in transgenic plants can interfere with different steps of virus infection, depending on the host–virus combination. However, there are several features that are shared in cases in which CP accumulation leads to virus resistance. One common observation of CPMR is limitation of resistance to the virus from which the CP gene is derived and to closely related viruses. This specificity suggests that host defense responses, like those that are involved in the development of nonspecific systemic acquired resistance, do not play a major role in CPMR.

In cases in which virion disassembly is affected in transgenic plants, the range of protection might depend on the ability of the accumulated CP to aggregate with CP subunits of the infecting virus. Such aggregation would lead to stabilization of the virion and prevent replication. The correlation of the degree of resistance of TMV CP-accumulating plants to other tobamoviruses with the degree of amino acid homology between their CPs might be an indication that this is the case.

Classical crossprotection seems sometimes to be caused by inhibition of viral uncoating. For example, infection of tobacco, tomato, or squash plants with the mild strain S of cucumber mosaic virus leads to resistance to CMV-P *(31)*. Crossprotection can be overcome by infection with CMV-P RNA. The same applies to CPMR to CMV in tobacco protoplasts *(28)*, and to TMV-infected *Nicotiana sylvestris* plants that can be superinfected with RNA or partially uncoated virions, but not virions of a necrotizing strain of TMV *(32)*. The presence of high concentrations of protecting virus has been shown to be more efficient in crossprotection between TMV strains than lower concentrations *(33)*. High concentrations of crossprotecting virus might result in higher concentrations of free CP that can function in stabilizing the virions of the superinfecting virus. The correlation of CP levels in transgenic tobacco with resistance to TMV suggests that both CPMP and crossprotection might in this case work by the same mechanism.

Reduced rate of virus accumulation in inoculated leaves and slower systemic spread are frequently observed in transgenic CP-accumulating plants. This could be a result of slower replication rates or interference with local and systemic virus transport. In many reports of CPMR, the presented data is not sufficient to clearly indicate which step in virus infection is inhibited. In cases in which CPMR can be overcome by inoculation with viral RNA, it is likely that protection is mainly because of inhibition of virion disassembly. However, additional steps can be affected. Replication rates of TMV in protoplasts are reduced after electroporation with up to 40 µg/mL TMV RNA. Transgenic plants accumulating the CMV CP can sometimes accumulate CMV in inoculated leaves, but no systemic spread occurs. In this case, there seems to be interference with either entry of the phloem or vascular long-distance transport.

## 8. Summary

Expression of viral CP genes in transgenic plants can lead to virus resistance by interference of either the transcript or the protein with virus infection Dependence of resistance on CP accumulation can be most convincingly shown by comparison of plants that accumulate CP with plants that accumulate a nontranslatable CP transcript. Even in cases in which CP accumulation is required, the degree of resistance does not always correlate with CP levels in transgenic plants.

In cases in which CPMR can be overcome by inoculation with viral RNA instead of virions, interference with virion disassembly is the likely cause of resistance. Classical crossprotection can also sometimes be overcome by RNA inoculation, and, in this case, appears to work by a similar mechanism. There is no evidence yet that CPMR is caused by a nonspecific plant defense response that might be triggered by the accumulating CP

Measurement of virus accumulation in protoplasts prepared from transgenic plants was used to show interference with early events of virus infection. There is no clear evidence yet for inhibition of local virus spread in transgenic plants. A reduced rate of virus accumulation in inoculated leaves can usually also be explained with reduced rate of replication. However, in the case of CPMR to CMV, it appears that early events, as well as systemic spread, are affected. Reduced vector transmission of virus infection from inoculated transgenic plants to nontransgenic plants has been observed. It is not known whether this is just a consequence of lower virus levels in the transgenic plants or whether direct interference with acquisition and transmission by the vector is also involved.

In addition to virion formation, CP can function in different ways in plant virus infections. Replication, long-distance spread, and vector transmission can also depend on the presence of CP. Expression of genes encoding nonfunctional CPs in transgenic plants can be tried in order to interfere with normal CP

function. Knowledge of CP function(s) in a particular plant–virus interaction will be useful to design gene constructs.

Since CP accumulation levels in transgenic plants do not always correlate with resistance, newly generated transgenic plant lines are now frequently tested for virus resistance before further characterization. However, if the objective of a transformation experiment is also to study the mechanism of CPMR, it is necessary to determine transcript and protein levels in the transgenic plants Gene constructs encoding nontranslatable and antisense CP transcripts should be included in the experiment. If possible, transgenic plants should be inoculated with virions and viral nucleic acid, and replication in isolated protoplasts should be determined.

## References


1. Fitchen, J. H. and Beachy, R. N. (1993) Genetically engineered protection against viruses in transgenic plants *Ann Rev Microbiol* **47,** 739–763
2 Powell-Abel, P, Nelson, R S , De, B , Hoffmann, N., Rogers, S G , Fraley, R T , and Beachy, R N (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene *Science* **232,** 738–743
3. Bol, J F , van Vloten-Doting, L , and Jaspars, E M J (1971) A functional equivalence of top component a RNA and coat protein in the initiation of infection by alfalfa mosaic virus *Virology* **46,** 73–85
4 Houwing, C. J. and Jaspars, E M. J. (1987) In vitro evidence that the coat protein is the programming factor in AlMV-induced RNA synthesis *FEBS Lett* **221,** 337–342
5. Atreya, P. L , Atreya, C D., and Pirone, T P (1991) Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus *Proc Natl Acad Sci USA* **88,** 7887–7891
6 Azzam, O , Frazer, J , de la Rosa, D , Beaver, J S , Ahlquist, P., and Maxwell, D P (1994) Whitefly transmission and efficient ssDNA accumulation of bean golden mosaic geminivirus require functional coat protein *Virology* **204,** 289–296
7 Briddon, R. W , Pinner, M S , Stanley, J , and Markham, P G (1990) Geminivirus coat protein replacement alters insect specificity *Virology* **177,** 85–94
8 Chen, B and Francki, R I B. (1990) Cucumovirus transmission by the aphid Myzuz persicae is determined solely by the viral coat protein *J Gen Virol* **71,** 939–944
9 McLean, M A., Campbell, R N , Hamilton, R I., and Rochon, D. M (1994) Involvement of the cucumber necrosis virus coat protein in the specificity of fungus transmission by Olpidium bornovanus *Virology* **204,** 840–842
10 Nelson, R S., Powell Abel, P., and Beachy, R N. (1987) Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. *Virology* **158,** 126–132.
11. Nejidat, A and Beachy, R N (1989) Decreased levels of TMV coat protein in transgenic tobacco plants at elevated temperatures reduce resistance toTMV infection. *Virology* **173,** 531–538

12 Powell, P A , Sanders, P R , Tumer, N , Fraley, R. T , and Beachy, R N (1990) Protection against tobacco mosaic virus infection in transgenic tobacco plants requires accumulation of coat protein rather than coat protein RNA sequences *Virology* **175,** 124–130

13. Carr, J P , Beachy, R N., and Klessig, D. F. (1989) Are the PR1 proteins of tobacco involved in genetically engineered resistance to TMV? *Virology* **169,** 470–473

14. Nejidat, A. and Beachy, R. N. (1990) Transgenic tobacco plants expressing a coat protein gene of tobacco mosaic virus are resistant to some other tobamoviruses *Mol. Plant–Microbe Interact* **3,** 247–251.

15. RegisterIII, J C. and Beachy, R N. (1988) Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology* **166,** 524–532

16. Reimann-Philipp, U. and Beachy, R N. (1993) Coat protein-mediated resistance in transgenic tobacco expressing the TMV coat protein from tissue specific promoters. *Mol Plant–Microbe Interact* **6,** 323–330.

17. Register III, J. C. and Beachy, R N. (1989) Effect of protein aggregation state on coat protein-mediated protection against tobacco mosaic virus using a transient protoplast assay *Virology* **173,** 656–663

18. Clark, W G , Fitchen, J , Nejidat, A , Deom, C M , and Beachy, R N (1995) Studies of coat protein-mediated resistance to tobacco mosaic virus (TMV) II Challenge by a mutant with altered virion surface does not overcome resistance conferred by TMV coat protein. *J. Gen. Virol* **76,** 2613–2617

19. Wisniewski, L A., Powell, P A., Nelson, R. S , and Beachy, R. N (1990) Local and systemic spread of tobacco mosaic virus in transgenic tobacco plants *Plant Cell* **2,** 559–567

20 van Dun, C M P, Overduin, B., van Vloten-Doting, L., and Bol, J. F (1988) Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not crossprotect against alfalfa mosaic virus infection *Virology* **164,** 383–389

21. Tumer, N E., Kaniewski, W , Haley, L., Gehrke, L , Lodge, J K., and Sanders, P (1991) The second amino acid of alfalfa mosaic virus coat protein is critical for coat protein-mediated protection *Proc Natl Acad Sci USA* **88,** 2331–2335

22. Tumer, N E , O'Connell, K M , Nelson, R. S , Sanders, P R , Beachy, R N , Fraley, R T., and Shah, D M (1987) Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants *EMBO J* **6,** 1181–1188.

23. Taschner, P E. M , van Marle, G., Brederode, F T , Tumer, N E., and Bol, J F (1994) Plants transformed with a mutant alfalfa mosaic virus coat protein gene are resistant to the mutant but not to the wild type virus. *Virology* **203,** 269–276

24 van Dun, C M P, Bol, J F , and van Vloten-Doting, L (1987) Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* **159,** 299–305

25 Hemenway, C , Fang, R X , Kaniewski, W K , Chua, N H., and Tumer, N E. (1988) Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA *EMBO J.* **7,** 1273–1280

26 Lawson, C , Kaniewski, W , Haley, L , Rozman, R , Newell, C , Sanders, P , and Tumer, N E (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar. resistance to potato virus X and potato Virus Y in transgenic Russet Burbank *Biotechnology* **8,** 127–134.

27 Lindbo, J A., Silva-Rosales, L., and Dougherty, W G (1993) Pathogen derived resistance to potyviruses. working, but why? *Semin Virol* **4,** 357–361

28 Okuno, T., Nakayama, M , and Furusawa, I (1993) Cucumber mosaic virus coat protein-mediated protection *Semin Virol* **4,** 357–361

29. Taliansky, M E and Garcia-Arenal, F. (1995) Role of cucumovirus capsid protein in long-distance movement within the infected plant *J Virol* **69,** 916–922

30 Ding, B , Haudenshield, J S , Hull, R J , Wolf, S , Beachy, R N , and Lucas, W J (1992) Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants *Plant Cell* **4,** 915–928

31 Dodds, J. A , Lee, S. Q , and Tiffany, M. (1985) Cross protection between strains of cucumber mosaic virus· effect of host and type of inoculum on accumulation of virions and double-stranded RNA of the challenge strain. *Virology* **144,** 301–309

32 Sherwood, J L and Fulton, R W (1982) The specific involvement of coat protein in tobacco mosaic virus cross protection *Virology* **119,** 150–158

33 Rezende, J A M and Sherwood, J L (1991) Susceptibility of dark green areas to superinfection leads to breakdown of cross protection between strains of tobacco mosaic virus *Phytopathology* **81,** 1490–1496

# 53

# Mechanisms of RNA-Mediated Resistance to Plant Viruses

**Peter de Haan**

## 1. Introduction

Viral diseases cause significant losses to almost all crops throughout the world. Infections with plant viruses can either cause direct yield losses or lead to unacceptable levels of postharvest damage to the crops. Besides measures to limit the virus incidence, the ultimate way to minimize losses caused by viral infections is the production of resistant varieties. This can be obtained by introgression of resistance genes from wild relatives, or by transformation of host plants with antiviral genes or viral sequences. Expression of viral coat protein (CP) in plants yields protection to the homologous virus. In addition to this CP-mediated protection (CPMR), a still growing number of reports deal with engineered virus resistance conferred by transgenic expression of viral transcripts, rather than proteins.

This chapter will describe this so-called RNA-mediated virus resistance (RMR) in more detail and discuss possible working mechanisms of this powerful technology.

## 2. Plant Resistance to Viruses

Most plants are resistant to or can hardly be infected by viruses. Nevertheless, plant viruses gained a significant impact in agriculture. First, breeding has usually been focused on gaining higher yields and improved product quality, rather than resistance to pathogens. Second, crop cultivations have been scaled up enormously, leading to monocultures, which makes the crops highly attractive for specific pathogens. Third, because of the intensified worldwide transport of plant material, viruses meet new host plants and vice versa. In contrast to infection with other pathogens, virus infections cannot be cured.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

Plant viruses can therefore only be controlled by taking a number of phytosanitary measures, including using pesticides, crop rotation, applying crossprotection, or, ultimately, breeding for resistance *(1,2)*.

Infection of plants with a mild strain of a given virus, which prevents infection by aggressive strains of the virus, has been referred to as classical crossprotection, or premunition. Commercial application of this method, however, has a number of disadvantages. Although the plant tolerates the mild protecting virus, infection still leads to reduced yields Moreover, the protecting virus can spread to neighboring crops, in which it may cause significant yield losses. In addition, coinfection with another virus might increase symptom development in crop plants. Finally, since viral RNA molecules easily undergo mutations during replication, aggressive strains may arise from the mild protecting strains It has long been debated whether accumulation of viral CP or replication of viral RNA is responsible for the observed interference with virus replication. The fact that virus mutants, which do not encode CP, are still capable of crossprotecting plants, supports the hypothesis that the replicating viral RNA is involved in crossprotection (*see* **Subheading 5.**) *(3)*

Plants find themselves protected from viral infections using passive or active defense mechanisms. Passive defense implies that the plant has factors that suppress, or lack factors that support, virus replication. These passive mechanisms may act on the level of transmission (often linked to insect resistance), multiplication (the plant is immune), transport (a virus causes a subliminal infection), and symptom development (the plant is tolerant). Passive virus resistance can be monogenic, but usually is multigenic and is then referred to as partial or horizontal resistance. Each separate gene contributes to the total level of protection.

In addition to passive defense mechanisms, plants may protect themselves from viral infections using active defense responses. Upon infection, resistant plants produce factors that suppress virus multiplication. The most important example of such modes of resistance is the hypersensitive response (HR) The HR can be explained by the classical gene-for-gene model, as outlined by Flor *(4)*, in which a product of an avirulence gene of the pathogen (a virus) is recognized by a receptor, e.g., the product of a plant resistance gene. Recognition of the pathogen leads to programmed cell death (apoptosis) at the site of infection, giving rise to the formation of local lesions and to induction of systemic acquired resistance (SAR) *(4)*. Active resistance is usually monogenic and is often referred to as cultivar or vertical resistance.

In breeding, introgression of horizontal sources of resistance is inconvenient. Since these sources are multigenic, breeding becomes time-consuming and labor-intensive. Moreover, genes contributing to the resistance can be closely linked to undesired agronomic traits. For these reasons, the use of ver-

tical sources of resistance is preferred. The general problem, however, with the application of such sources of resistance is often their durability. Since the active resistance mechanisms are triggered upon recognition of the virus by the plant, the resistance can be overcome by new viral strains or pathotypes, which have single, or few, point mutations in their genomes. For example, pepper varieties containing the L1, L2, L3, or L4 tobamovirus resistance genes, are frequently infected with novel pepper mild mottle tobamovirus pathotypes Resistance-breaking pathotypes already appeared in the breeding phase, during the introgression process of an L resistance gene *(5)*.

As mentioned, many crops lack resistance genes to viruses. This is well-illustrated in the *Cucurbitaceae* The cultivation of cucurbit crops in the open field is severely affected by a number of potyviruses, closteroviruses, and cucumber mosaic cucumovirus. Unfortunately, besides the cultivated cucurbits, their wild relatives are often also susceptible to these viruses. Hence, suitable sources of resistance, to be introgressed into cultivated cucurbits, are absent. This illustrates that in some crops, novel strategies to obtain virus resistance are urgently required.

## 3. Engineered Resistance to Plant Viruses

Over the past decade, our knowledge of plant transformation technology and molecular virology has increased rapidly. This has opened the way to genetically engineer virus resistance Protection to viruses has been reported by expression of a number of antiviral agents in plants, including ribozymes, 2'–5' oligoadenylate synthetase, double-stranded ribonucleases (dsRNases), ribosomal inhibiting proteins (RIPs), CP-ribonuclease (CP-RNase) fusion proteins, (pl)antibodies, and antibody-enzyme (abzyme) fusion proteins *(6,7)*. At least some of these approaches look promising, but it is beyond the scope of this chapter to describe these strategies in more detail

Engineered plant-derived resistance may also be obtained by transgenic expression of durable vertical resistance genes in other crop plants In this respect, one can imagine that expression of the highly durable Tm-$2^2$ tobamovirus resistance gene (from tomato) in pepper could serve as an alternative to the nondurable L resistance genes in this crop, provided that this gene operates similarly in pepper *(2,5)*.

Pathogen-derived resistance comprises expression of viral genes in plants. Transgenic expression of symptom-attenuating satellite RNA molecules in plants, or expression of defective-interfering (DI) RNA molecules, leads to a certain level of tolerance in host plants. The tolerance is conferred by coreplication of these symptom-attenuating RNA molecules with the corresponding helper virus. Since only a few plant viruses support satellite RNAs and DI RNAs, these approaches are generally not applicable.

**Table 1**
**A Selected List of Reports on Pathogen-Derived Resistance to Viruses**

| Virus | Supergroup | DNA construct | | | | | Ref |
|---|---|---|---|---|---|---|---|
| | | CP | CP RNA | RdRp | MP | Other | |
| AlMV | 1 | + | | + | | | *10,11* |
| CMV | 1 | + | | + | | | *12,13* |
| CPMV | 4 | | | + | + | | *14* |
| CRSV | 2 | | | + | | | *15* |
| PEBV | 1 | | | + | | | *16* |
| PLRV | 5 | | + | | | | *17* |
| PVS | 1 | + | | | | | *18* |
| PVX | 1 | + | | + | | | *19,20* |
| PVY | 4 | | + | + | | + | *21–23* |
| TEV | 4 | | + | | | | *24* |
| TMV | 1 | + | | + | | | *25,26* |
| TRV | 1 | + | | | | | *10* |
| TSV | 1 | + | | | | | *27* |
| TSWV | 3 | | + | | + | | *28,29* |

The pluses refer to the viral genes used for transformation CP, coat protein, CP RNA, untranslatable CP RNA, RdRp, RNA-dependent RNA polymerase, MP, movement protein The viruses are abbreviated as follows AlMV, alfalfa mosaic alfamovirus, CMV, cucumber mosaic cucumovirus, CPMV, cowpea mosaic comovirus, CRSV, cymbidium ringspot tombusvirus, PEBV, pea early browning tobravirus, PLRV, potato leaf roll luteovirus; PVS, potato S carlavirus, PVX, potato X potexvirus, PVY, potato Y potyvirus, TEV, tobacco etch potyvirus, TMV, tobacco mosaic tobamovirus, TRV, tobacco rattle tobravirus, TSV, tobacco streak ilarvirus, TSWV, tomato spotted wilt tospovirus The viral Supergroups are denoted, with permission, after **ref.** *29* 1, α-like, 2, Carmo-like, 3, (–)-stranded, 4, Picorna-like, 5, Sobemo-like

Expression of mutated viral genes is a promising novel technology. Transformation of tobacco plants with a mutant movement protein (MP) of tobacco mosaic tobamovirus inhibited systemic infection of the transgenic plants by a broad scala of plant viruses. Apparently, this crippled protein carries out part of its function, but blocks another step in virus movement, and thereby interferes with cell-to-cell spread of viruses *(8)*.

Accumulation of viral CP confers resistance to the virus from which the CP gene was derived (*see* Chapters 3 and 52). This CPMR is established technology and it is expected that in the very near future the first transgenic virus-resistant products based on CP technology will be released to the market *(9)*.

**Table 1** is a list of reports dealing with pathogen-derived resistance. The reports in which it has unequivocally been demonstrated that accumulation of CP confers virus resistance have a plus under CPMR. Remarkably, all viruses

for which this is the case, belong to the α-like supergroup of RNA viruses. Viruses belonging to this supergroup express some of their genes via translational readthrough and by translation from subgenomic mRNAs *(30)*. The viral RNA molecules are probably transported from cell to cell as RNA–MP complexes, through plasmodesmata, with altered size-exclusion limits *(30)* Another important supergroup of plant viruses is the Picorna-like supergroup Members of this supergroup express their genes via proteolytic cleavage of precursor proteins *(30)*. Cell-to-cell transport takes place as virus particles, via tubules through the plasmodesmata. These so-called desmotubules are most likely built up by viral MP *(31)*.

It has been proposed that the transgenically produced CP inhibits the cotranslational disassembly of invading virus particles. This implies that CPMR should also have been reported for other than α-like viruses This seems not to be the case. For this reason, it seems more likely that the transgenically produced CP mainly inhibits other and later steps in the viral infection process, such as RNA replication–transcription and/or viral cell-to-cell movement, as has been suggested by some authors *(32)*. For the α-like viruses, CP is not required for cell-to-cell movement, which might be an indication that the presence of transgenically produced CP in plant cells interferes with replication–transcription of viral RNAs. In analogy to (–)-stranded viruses, it can be assumed that the amounts of CP present in the cytoplasm of plant cells infected with α-like viruses determine whether the viral RdRp is in the replicative or transcriptive mode. If this is indeed the case, then the presence of transgenically produced CP interferes with the replication-to-transcription switch, leading to abortive replication and virus resistance *(33)*. On the other hand, it seems obvious that, for the Picorna-like viruses, which move from cell to cell as virus particles, the presence of relatively small amounts of transgenically produced CP does not have any effect on replication, viral cell-to-cell movement, and systemic spread.

## 4. RNA-Mediated Resistance to Plant Viruses

The first indications suggesting that other than protein-encoding sequences of viral origin were capable of conferring resistance came from experiments in which replicase or RNA-dependent RNA polymerase (RdRp) sequences of potato X potexvirus, tobacco mosaic tobamovirus, and pea early browning tobravirus were expressed in transgenic tobacco plants The manifestation of the obtained resistance differed completely from that conferred by CP *(6,7)*. Some, but not all, transformants showed extreme resistance to the homologous virus, and the levels of resistance were not correlated with the amounts of transgenically produced proteins or transcripts (*see* **Table 2**)

**Table 2**
**Some Properties of CP- and RNA-Mediated Virus Resistance**

| Virus resistance | CP-mediated | RNA-mediated |
|---|---|---|
| Viral gene involved. | CP gene | RdRp, MP, or other genes |
| Initial interactions· | Protein–protein or protein–RNA | RNA–RNA |
| Possible working mechanisms· | Blocking of movement and/or translation | RNA degradation |
| Capacity | Dosis-dependent | Dosis-independent |
| Spectrum | Broad (against members of a genus) | Narrow (against homologous viruses) |
| Specificity | α-like supergroup | All supergroups |

CP, coat protein, RdRp, RNA-dependent RNA polymerase, MP, movement protein

Expression of a truncated RdRp gene of cucumber mosaic cucumovirus conferred immunity to the homologous virus *(13)*. Transgenic tobacco plants harboring an untranslatable RdRp gene or an antisense RdRp gene also show resistance to CMV. On the contrary, DNA constructs based on CMV RNA-3 are not capable of conferring resistance (unpublished observations). Concurrently with these reports in which RdRp sequences are used, many more reports appeared dealing with this type of pathogen-derived resistance. Transgenic tobacco plants expressing untranslatable CP cistrons of tobacco etch potyvirus and potato Y potyvirus show immunity to the respective potyviruses *(21,24)*. Since untranslatable RNAs are capable of conferring virus resistance, this clearly indicates that the transgenic viral transcripts are involved in the resistance. Therefore, this type of engineered resistance has been denoted RNA-mediated virus resistance (RMR) The number of (+)-strand viruses for which RMR has been obtained is still expanding, and at this moment alfamo-, carmo-, como-, cucumo-, potex-, poty-, tobamo-, tobra-, and tombusviruses are included (*see* **Table 1**)

Transformation of host plants with (untranslatable) nucleoprotein gene sequences of tomato spotted wilt tospovirus, a (–)-stranded RNA virus belonging to the *Bunyaviridae*, confers high levels of virus resistance *(28)*. This is a clear demonstration that RMR is not limited to the (+)-stranded viruses.

Transformation of tomato plants with RdRp sequences of tomato golden mosaic geminivirus, a virus with a circular DNA genome, yielded transgenic plants that show a significant reduction in symptom development following inoculation with the homologous virus *(34)*. This at least suggests that RMR might also work for viruses with DNA genomes

Remarkably, constructs derived from (+)-stranded viruses, capable of conferring virus resistance, contain replicase or RdRp sequences, or sequences corresponding to viral RNAs encoding (subunits of) the RdRp. This is the reason why, in the past, the resistance was often referred to as replicase-mediated virus resistance *(13,15,21,26)*.

At least for some (+)-stranded viruses, it has been shown that cotranslational disassembly is the initial step in the viral infection process. In general, this leads to the production of RdRp, which is necessary for replication and/or subsequent transcription, and hence expression of the other viral genes. Obviously, resistance can be obtained by interference with the production of RdRp, blocking the first and critical process in virus replication. In addition, recently it has been reported that resistance to the Picorna-like viruses and the (–)-stranded viruses can also be obtained by expression of genes involved in cell-to-cell movement *(14,29)*. As mentioned in **Subheading 3.**, the Picorna-like viruses move from cell to cell via desmotubules consisting of viral MP. For tomato spotted wilt tospovirus, it has recently been demonstrated that viral nucleocapsids also move from cell to cell via desmotubules *(35)*. It can therefore be assumed that inhibition of the expression of MP genes of these viruses prevents the formation of desmotubules, thereby inhibiting cell to cell and systemic spread.

For viruses that move from cell to cell via plasmodesmata with altered size-exclusion limits, resistance cannot be obtained by interference with the expression of MP genes. Obviously, the production of very small amounts of MP is sufficient for virus transport, and hence for normal systemic infection.

Among the plant viruses, potyviruses, belonging to the Picorna-like supergroup, are real outsiders in a number of molecular aspects. The same holds for engineered potyvirus resistance, since it has been shown that sequences derived from different parts of the potyviral genome are capable of conferring resistance *(21,22,36)* This might be related to the fact that potyviruses have an undivided RNA genome, encoding a single polyprotein. Hence, resistance can be obtained by interference with the production of this polyprotein, thereby preventing the synthesis of (–)-strand RNA molecules and other processes, including viral cell-to-cell movement.

Despite numerous attempts, only a single clear report appeared on RMR against a phloem-restricted virus, potato leaf roll luteovirus *(17)*. So far, pathogen-derived resistance has not been obtained to other important luteoviruses, such as beet western yellows and barley yellow dwarf luteovirus. Luteo- and closteroviruses obligatorily replicate in phloem tissue including sieve tubes, companion cells, and so on. Since the sieve tubes lack nuclei, it might be that the inhibiting transgenic RNAs (or CPs) are simply not present in cells where these viruses replicate. This implies that other, more novel, strategies need to

be developed to obtain resistance against these economically important groups of plant viruses.

In conclusion, accumulating evidence is available that RMR can be obtained to the vast majority of plant viruses, including the (+)- and (–)-stranded viruses, and possibly even DNA viruses.

## 5. Working Mechanisms of RNA-Mediated Virus Resistance

Despite intensive research, our knowledge on the exact molecular mechanisms by which RMR works is still fragmented. This information is essential in order to predict the long-term stability of such engineered sources of resistance and to further improve this relatively novel technology.

One remarkable property of RMR is that it is only active against homologous viruses. The resistance is overcome by more distantly related viruses. A general rule of thumb is that at least 90% nucleotide sequence homology between transgene and corresponding viral gene is required to obtain resistance *(13,26,28)*. For this reason, it can be assumed that engineered virus resistance cannot simply be overcome by pathotypes with single or few point mutations in their genome. Assuming that the transgenes behave stably over generations, it can be anticipated that RMR will confer durable virus resistance to crops.

Experiments performed by Lindbo and Dougherty *(24)* and Lindbo et al. *(37)*, using transgenic tobacco plants resistant to tobacco etch or potato Y potyvirus, provided the first hints that RMR is conferred by sequence-specific RNA degradation in the cytoplasm. In vitro run-on labeling experiments using potyvirus-resistant tobacco plants demonstrated that the rate of transcription in the nuclei of resistant plant cells is high, compared with that in nuclei of susceptible transgenic plant cells. In contrast, in the resistant plant cells the steady-state levels of transgenic mRNA are low, sometimes even lower than those in susceptible transgenic plants. This suggests that the transgenic mRNAs (and most likely, also, the homologous RNAs of invading viruses) are selectively and rapidly degraded in the cytoplasm of resistant plant cells. These findings have recently been confirmed for several other plant–virus combinations *(20,36)*.

As mentioned in the previous subheading, resistance can be obtained by expression of RdRp, MP (or other viral sequences, in case of potyviruses) in plants. However, so far, it has remained unknown which parts of the genes are capable of conferring resistance. Although speculative, it appears that the 5' leader and 3' trailer sequences, and the length of the transgenically produced mRNAs, play important roles.

For most viruses, antisense RNAs are also capable of conferring virus resistance, but this does not seem to hold for all plant viruses. Possibly, the

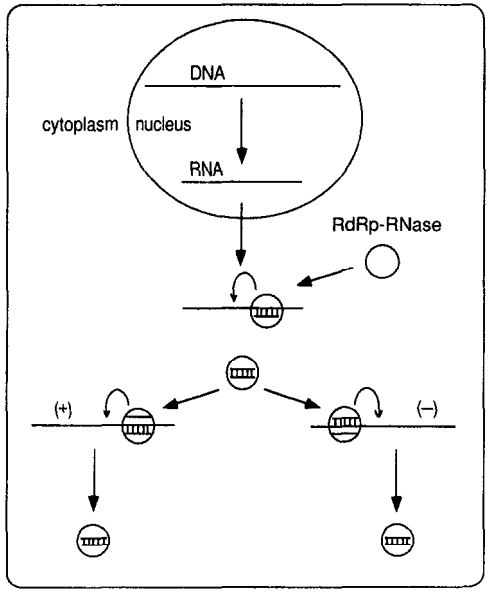



Fig. 1 Model to explain RMR The transgene is transcribed in the nucleus and the mRNAs are transported to the cytoplasm. Some mRNAs are recognized by an RNA-dependent RNA polymerase (RdRp)–RNase complex, which synthesizes short complementary RNA molecules The transgenic mRNAs are rapidly degraded and the RdRp–RNase complex uses the short duplex RNA to tag homologous (+) or possibly complementary (–) RNA sequences, and subsequently degrades them

transgenically produced antisense RNAs were too short in these experiments, or the target viral RNAs, e.g., the minus-sense RNAs, somehow are protected and are not capable of interacting with the transgenic RNA *(38)*.

Taking all data on RMR together, it seems that, at least for most viruses, the polarity of the transgenic transcripts is not important for their capability to confer resistance. This leads to the hypothesis that RMR and antisense inhibition of gene expression might operate via similar or even identical mechanisms.

**Figure 1** is a schematic representation of a possible model to explain RMR. In the transgenic virus-resistant plant cells, the transgenic mRNAs are recognized by a cytoplasmic host factor This factor can be conceived as a RdRp-RNase complex, since it first synthesizes a short complementary RNA, and subsequently degrades the single-stranded parts of the mRNAs. With the help of the remaining short nucleic acid duplex, the complex is able to tag and degrade sequences homologous, or possibly complementary, to the transgenic mRNAs.

A number of observations support this model, with a cytoplasmic RdRp-RNase complex as the major active component. First, many eukaryotic RNA polymerases and RNases contain small nucleic acid fragments. Second, RdRp activity is occasionally present in plant cells. Third, a host-encoded RdRp could be purified from virus-infected plant tissue *(39,40)*.

Experiments on transgenic expression of pigmentation genes in *Petunia (41)*, on softening enzymes in tomato *(42)*, and on β-1,3-glucanase in tobacco *(43)*, revealed that cosuppression of gene expression results from an increase in RNA turnover in the cytoplasm. In addition, the carotenoid biosynthesis in tobacco plants can be modified by inoculation with RNA viral vectors containing the tomato phytoene synthase gene, encoding the key enzyme in the carotenoid biosynthesis, under the transcriptional control of a tobamovirus subgenomic promoter *(44)*. These experiments clearly demonstrate that cosuppression and RMR reflect identical or similar posttranscriptional and cytoplasmic events. Both virus-resistant and cosuppressing plant cells contain a genetically stable memory for specific RNAs, which are readily degraded upon recognition *(45)*.

Besides cosuppression, the proposed RMR model can also be adapted to explain antisense inhibition of gene expression. Moreover, classical plant virological phenomena, such as virus crossprotection (**Subheading 2.**), or even mosaic symptoms with light–dark-green islands *(36)*, might be explained by this model.

An intriguing observation that still needs to be explained is the fact that, in a given transformation experiment, not all different transgenic plants show the virus-resistant or cosuppression phenotype. In tobacco, in our hands, on average 1 out of 10–20 transformants shows the desired phenotype This frequency, however, differs from crop to crop, and, moreover, it has become clear that it is dependent on many other factors. First, the position of transgene copies on the host chromosomes is of importance. Host sequences flanking the T-DNA inserts have an influence on the levels and patterns of expression of the transgenes on the T-DNA. Second, the number of inserted T-DNA copies and the orientation toward each other also appear to affect the frequency of cosuppression *(41)*.

From the run-on transcription experiments, it can be deduced that there is a tendency that only highly active copies activate this sequence-specific RNase activity *(20,24,36,37)*. However, it has been demonstrated that promoterless DNA constructs are also capable of cosuppressing expression of host homologs *(41)*. These observations suggest that, not the levels of transcription of the transgenes, but the properties of the transgene loci may be responsible for the observed phenotype.

In summary, RMR or cosuppression is caused by overaccumulation of transgenic transcripts or by accumulation of transcripts with aberrant structures.

The imposed sequence-specific RNase activity might therefore be regarded as a natural host mechanism involved in mRNA turnover, or in removal of aberrant mRNA molecules. Future research will be dedicated to characterizing this cytoplasmic RNase activity and to determining its precise role in RMR. This knowledge will undoubtedly lead to further improvement of the potentially powerful RMR technology.

## Acknowledgments

I would like to thank Marcel Prins and Mart van Grinsven for our stimulating discussions and for critically reading this manuscript.

## References

1. Fraser, R. S. S. (1986) Genes for resistance to plant viruses. *Crit. Rev. Plant Sci.* **3,** 257–294.
2. Fraser, R. S. S. (1992) The genetics of plant-virus interactions: implications for plant breeding. *Euphytica* **63,** 175–185.
3. Sarkar, S. and Smitamana, P. (1981) A proteinless mutant of tobacco mosaic virus: evidence against the role of a viral coat protein for interference. *Mol. Gen. Genet.* **184,** 185–159.
4. Atkinson, M. M. (1993) Molecular mechanisms of pathogen recognition by plants. *Adv. Plant Pathol.* **10,** 35–64.
5. Tobias, I., Fraser, R. S. S., and Gerwitz, A. (1989) The gene-for-gene relationship between *Capsicum annuum* L. and tobacco mosaic virus: effects on virus multiplication, ethylene synthesis and accumulation of pathogenesis-related proteins. *Physiol. Mol. Plant Pathol.* **35,** 271–286.
6. Wilson, T. M. A. (1993) Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. *Proc. Natl. Acad. Sci. USA* **90,** 3134–3141.
7. Hull, R. (1994) Resistance to plant viruses: obtaining genes by non-conventional approaches. *Euphytica* **75,** 195–205.
8. Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J., and Beachy, R. N. (1993) A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *Plant J.* **4,** 959–970.
9. Beachy, R. N. (1990) Coat protein-mediated resistance against virus infection. *Ann. Rev. Phytopathol.* **28,** 451–474.
10. Van Dun, C. M. P., Bol, J. F., and Van Vloten-Doting, L. (1987) Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* **159,** 299–305.
11. Brederode, F. T., Taschner, P. E. M., Posthumus, E., and Bol, J. F. (1995) Replicase-mediated resistance to alfalfa mosaic virus. *Virology* **207,** 467–474.
12. Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R.-X., Chua, N.-H., and Tumer, N. E. (1988) Viral protection in transgenic plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Biotechnology* **6,** 549–557.

13. Zaitlin, M., Anderson, J. M., Perry, K. L., Zhang, L., and Palukaitis, P. (1994) Specificity of replicase-mediated resistance to cucumber mosaic virus. *Virology* **201,** 200–205.
14. Sijen, T., Wellink, J., Hendriks, J., Verver, J., and Van Kammen, A. (1995) Replication of cowpea mosaic virus RNA1 or RNA2 is specifically blocked in transgenic *Nicotiana benthamiana* plants expressing full-length replicase or movement protein genes. *Mol. Plant–Microbe Interact.* **8,** 340–347.
15. Rubino, L. and Lupo, R. (1993) Resistance to *Cymbidium* ringspot tombusvirus infection in transgenic *Nicotiana benthamiana* plants expressing a full-length viral replicase gene. *Mol. Plant–Microbe Interact.* **6,** 729–734.
16. MacFarlane, S. A. and Davies, J. W. (1992) Plants transformed with a region of the 201-kilodalton replicase gene from pea early browning virus RNA1 are resistant to virus infection. *Proc. Natl. Acad. Sci. USA* **89,** 5829–5833.
17. Kawchuk, L. M., Martin, R. R., and McPherson, J. (1991) Sense and antisense RNA-mediated resistance to potato leafroll virus in Russet Burbank potato plants. *Mol. Plant–Microbe Interact.* **3,** 247–253.
18. MacKenzie, D. J., Tremaine, J. H., and McPherson, J. (1991) Genetically engineered resistance to potato virus S in potato cultivar Russet Burbank. *Mol. Plant–Microbe Interact.* **5,** 34–40.
19. Hemenway, C., Fang, R.-X., Kaniewski, W. K., Chua, N.-H., and Tumer, N. E. (1988) Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.* **7,** 1273–1280.
20. Mueller, E., Gilbert, J., Davenport, G., Brigneti, G., and Baulcombe, D. C. (1995) Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant J.* **7,** 1001–1013.
21. Audy, P., Palukaitis, P., Slack, S. A., and Zaitlin, M. (1994) Replicase-mediated resistance to potato virus Y in transgenic tobacco plants. *Mol. Plant–Microbe Interact.* **7,** 15–22.
22. Maiti, I. B., Murphy, J. F., Shaw, J. G., and Hunt, A. G. (1993) Plants that express a potyvirus proteinase gene are resistant to virus infection. *Proc. Natl. Acad. Sci. USA* **90,** 6110–6114.
23. Vardi, E., Sela, I., Edelbaum, O., Livneh, O., Kuznetsova, L., and Stram, Y. (1993). Plants transformed with a cistron of potato virus Y protease (NIa) are resistant to virus infection. *Proc. Natl. Acad. Sci. USA* **90,** 7513–7517.
24. Lindbo, J. A. and Dougherty, W. G. (1992) Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. *Virology* **189,** 725–733.
25. Powell-Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232,** 738–743.
26. Donson, J., Kearney, C. M., Turpen, T. H., Khan, I. A., Kurath, G., Turpen, A. M., Jones, G. E., Dawson, W. O., and Lewandowski, D. J. (1993) Broad resistance to tobamoviruses is mediated by a modified tobacco mosaic virus replicase transgene. *Mol. Plant–Microbe Interact.* **6,** 635–642.

27. Van Dun, C. M. P., Overduin, B., Van vloten-Doting, L., and Bol, J. F. (1988) Transgenic tobacco expressing tobacco streak virus ormutated alfalfa mosaic virus coat protein gene does not cross-protect against alfalfa mosaic virus infection. *Virology* **164,** 383–389.
28. De Haan, P., Gielen, J. J. L., Prins, M., Wijkamp, M. G., Van Schepen, A., Peters, D., Van Grinsven, M. Q. J. M., and Goldbach, R. W. (1992) Characterisation of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. *Biotechnology* **10,** 1133–1137.
29. Prins, M., Ismayadi, C., De Graauw, W., De Haan, P., and Goldbach R. (1997) RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NSm gene sequences. *Plant Mol. Biol.* **33,** 235–243.
30. Goldbach, R. and De Haan, P. (1994) RNA viral Supergroups and the evolution of RNA viruses, in *The Evolutionary Biology of Viruses* (Morse, S. S., ed.), Raven, New York, pp. 105–119.
31. Deom, C. M., Lapidot, M., and Beachy, R. N. (1992) Plant virus movement proteins. *Cell* **69,** 221–224.
32. Osbourn, J. K., Watts, J. W., Beachy, R. N., and Wilson, T. M. A. (1989) Evidence that nucleocapsid disassembly and a later step in virus replication are inhibited in transgenic tobacco protoplasts expressing TMV coat protein. *Virology* **172,** 370–373.
33. Goldbach, R. and De Haan, P. (1993) Prospects of engineered forms of resistance against tomato spotted with virus. *Sem. Virol.* **4,** 381–387.
34. Bejarano, E. R., Day, A. G., Paranjape, V., and Lichtenstein, C. P. (1992) Antisense genes as tools to engineer virus-resistance in plants. *Biochem. Soc. Trans.* **20,** 757–761.
35. Kormelink, R., Storms, M., Van Lent, J., Peters, D., and Goldbach, R. (1994) Expression an subcellular localization of the NSm protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. *Virology* **200,** 56–65.
36. Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A., and Dougherty, W. G. (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation and fate of nonessential RNAs. *Plant Cell* **6,** 1441–1453.
37. Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M., and Dougherty, W. G. (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell* **5,** 1749–1759.
38. Bourque, J. E. (1995) Antisense strategies for genetic manipulations in plants. *Plant Sci.* **105,** 125–149.
39. Schiebel, W., Haas, B., Marikovic, S., Klanner, A., and Sänger, H. L. (1993) RNA-directed RNA polymerase from tomato leaves. II. catalytic *in vitro* properties. *J. Biol. Chem.* **268,** 11,858–11,867.
40. Dorssers, L. (1983) RNA-dependent RNA polymerases from cowpea mosaic virus-infected cowpea leaves. PhD Thesis, Wageningen Agricultural University, The Netherlands.
41. Van Blokland, R., Van der Geest, N., Mol, J. N. M., and Kooter, J. M. (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J.* **6,** 861–877.

42. Grierson, D., Fray, R. G., Hamilton, A. J., Smith, C. J. S., and Watson, C. F. (1991) Does co-suppression of sense genes in transgenic plants involve antisense RNA. *Trends Biotechnol.* **9,** 122,123.
43. De Carvalho Niebel, F., Frendo, P., Van Montagu, M., and Cornelissen, M. (1995) Post-transcriptional co-suppression of β-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. *Plant Cell* **7,** 347–358.
44. Kumagai, M. H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. K. (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. USA* **92,** 1679–1683.
45. Matzke, M. A. and Matzke, A. J. M. (1995) How and why do plants inactivate homologous (trans)genes. *Plant Physiol.* **107,** 679–685.

# 54

# Detection of Risks Associated with Coat Protein Transgenics

**Roger Hull**

## 1. Introduction

Recent advances in the understanding of the molecular mechanisms of how viruses function and how they interact with plants have led to the development of various nonconventional approaches to protection of plants against viruses. Many of these approaches involve the introduction of viral or virus-based sequences into the plant's genome. Expression of these sequences then interferes with one or more of the viral functions, thus giving some protection against the virus. This topic has attracted considerable attention and has been reviewed several times previously (*see* **refs.** ***1–16***). The viral genes most frequently used to provide protection are those for the coat protein (CP), the viral replicase, and the cell-to-cell movement protein (*see* reviews mentioned above for details). In the case of CP, protection is often given by the unmodified gene product. However, most other gene products are used in a form modified to affect normal functioning. There is increasing evidence that in some situations it is the expressed RNA, and not the gene product, that gives the protection (*see* Chapter 53).

Many of these transgenic plant lines, and especially those expressing CP, have reached the stage of field testing for the efficacy of protection, and are even being more generally field-released. This raises the question of possible risks that could arise on general field release, a topic that has previously been discussed by Hull ***(5–7)***, Hull and Davies ***(12)***, de Zoeten ***(17)***, and Tepfer ***(18)***. Despite these discussions, the issue has not been fully resolved, and various other aspects are being raised. This chapter discusses some of the ways by which potential risks, especially the use of CP genes, could be recognized and circumvented.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor © Humana Press Inc , Totowa, NJ

### 1.1. Potential Risks on Field Release

The two main areas of concern for most transgenic plants are possible adverse effects of the expression of the transgene on animals feeding on the plant and spread of the transgene into wild plant species. There is no evidence that the expression of plant viral sequences could be detrimental to man or other animals feeding on transgenic plants; in fact, virus-infected plants have been eaten for millennia. Furthermore, the lack of recognized allergy problems caused by pollen from virus-infected plants indicates that the expression of viral transgenes is unlikely to cause any problems in this area.

The area of concern specific to viral transgenes is the potential risks on any interactions between the viral or virus-related sequences being expressed from the transgene and another virus superinfecting that plant. Three main scenarios are usually considered: synergism, recombination, and heteroencapsidation.

### 1.2. Synergism

The possible synergistic effect of a viral transgene on a superinfecting virus can have two manifestations. It could enhance the symptoms of the superinfecting virus. Such synergism between viruses is well-known, for instance, between potato virus X (PVX) and various potyviruses in tobacco and tomato (tomato streak). A recent report *(19)*, demonstrating a synergistic effect of the expression of the 5' proximal sequence of tobacco vein mottling potyvirus as a transgene on infection with PVX, highlights that this problem has to be kept in mind. An alternative synergistic effect is that the expression of the transgene could mobilize a superinfecting virus that normally would be localized to the site of infection (subliminal infection). At present, there is no evidence of transgenically expressed CP giving any synergistic effect.

No detailed protocol is necessary for detecting synergistic reactions. Transgenic and nontransgenic plant lines should be inoculated with viruses that they are expected to encounter in the field, and the symptoms produced should be compared. More severe symptoms in the transgenic line(s) could be an indication of synergistic effects. However, they may also be caused by somaclonal variation caused by the transformation process.

### 1.3. Recombination

Three sorts of recombination have been recognized *(20)*: homologous with crossovers between related RNAs at precisely matched sites, aberrant homologous with crossovers between related RNAs not at corresponding sites, and nonhomologous with crossovers between unrelated RNAs at noncorresponding sites. There is considerable evidence for extensive recombination in RNA viruses (*see* **refs.** *20* and *21* for details), and probably all three mechanisms have been involved at one time or another. It is generally considered that

recombination plays an important role in the evolution of RNA viruses (*see* **refs. *20–23***). Evidence is now forthcoming of recombination between superinfecting viral RNA and RNA expressed from a transgene ***(24)*** through the aberrant homologous recombination mechanism. The finding of recognizable host RNA sequences within viral RNAs ***(25,26)*** is suggestive of nonhomologous recombination.

All the experimentation on recombinants between plant virus sequences has been done in controlled laboratory situations. It is difficult to devise detailed protocols for the detection of recombinants produced in the field. However, the basic principles are straightforward. One has to have a system to address the question as to whether the transgene sequence is covalently linked to the superinfecting viral sequence. This can be done by polymerase chain reaction (PCR) with primers to the transgene sequence (easy to devise) and to the superinfecting virus sequence (much more difficult to predict). If a recombinant is found, it has to be characterized to demonstrate that it is infective and viable. Only then can it be considered to be a potential risk.

### *1.4. Heteroencapsidation*

This involves the superinfection of a plant expressing the CP of a virus, say virus A, by an unrelated virus B. Heteroencapsidation is the encapsidation of the genome of virus B by the CP of A, thereby conferring on virus B properties of virus A. There are several examples of heteroencapsidation in transgenic plants, both between viruses of the same group ***(27,28)***, and between unrelated viruses ***(29)***. The main property of CP that is considered is that of vector transmission characteristics. However, there is increasing evidence that CPs are involved in long distance viral movement around infected plants, and heteroencapsidation could enhance the movement of a superinfecting virus that did not normally move systemically (*see* **Subheading 1.2.**).

The discussion of heteroencapsidation has focused on superinfecting viruses. However, there is the possibility that heteroencapsidation of retrotransposons could present a problem. Retrotransposons are a major class of transposable elements whose structure resembles the integrated copies of retroviruses, and which are considered to be important in evolution (*see* **ref. *30***). The *Tyl-copia* group of retrotransposons is widespread in plant genomes ***(31–33)***, and it has been suggested that there might be horizontal transmission between species ***(31)***. Sequencing has shown that most copies of the *Tyl-copia* retrotransposons in plants are mutated, so they would not be active. However, several active ones capable of retrotransposition have been described ***(30,34–37)*** and presumably replicate, as do all retrotransposable elements, via RNA. Among the factors that activate plant retrotransposons is tissue-culture, a process involved in transformation ***(37)***. This raises the possibility that introduction of the CP

transgene could activate retrotransposon RNA, which becomes heteroencapsidated and transmitted horizontally to other species.

### 1.5. Risk Reduction and Control

The main question to be addressed is whether the risk on field release of the transgenic plant is significantly more than the risk from the nontransgenic plant. To answer this, a wide range of controlled release experiments have been performed.

It is likely that it will take some time for a full risk assessment on the viral transgenic plants to be performed and commercial and other pressures will be very strong for field release. There are two approaches to risk reduction and control that can be put into effect relatively soon. One is to use biological containment *(7)*. In this approach, the region(s) of the transgene giving the undesirable properties are deleted, while retaining those that give the desirable protection property. A good example of this approach is found in the potyviral CP, which has an amino acid triplet (asp, ala, gly; DAG), which is involved in the interactions with the aphid vector *(38–40)*. Mutations of this motif, or its removal (which does not affect the protection ability of the CP; **ref.** *41*), would render heteroencapsidation with the transgene unable to confer aphid transmissibility on the superinfecting virus. Much more difficult is to avoid recombination, but targeted research on this may reveal methods.

The second approach is to design methods for monitoring the effects of field release. For small-scale releases, it is relatively easy to design monitoring procedures for analyzing pollen flow into related weeds and for detecting heteroencapsidants or recombinants. This will be much more difficult, if not impossible, for large-scale releases, in which the approach should be to educate farmers and extension service personnel to identify any unusual event that might be associated with the transgenic plants. This will be the challenge for the future.

### 1.6. Protocol for Detecting Heteroencapsidation

The basis of this protocol, modified from that of Candelier-Harvey and Hull *(29)*, is to immunecapture the heteroencapsidant, using an antiserum to the transgenically expressed CP, and to detect any encapsidated superinfecting viral (or retrotransposon) RNA by PCR.

## 2. Materials (All chemical Analar grade from BDH, Poole, UK, or Sigma-Aldrich, Poole, UK)

### 2.1. Solutions

1 Coating buffer: 1.59 g $Na_2CO_3$, 2 93 g $NaHCO_3$, 1 L $H_2O$, pH 9 6
2 Phosphate-buffered saline (PBS): 4 6 g $Na_2HPO_4$, 3 45 g $NaH_2PO_4$ $H_2O$, 8 5 g NaCl, 1 L $H_2O$, pH 7 2

3 PBS-T Add 0.5 mL Tween-20 to 1 L of PBS.
4. PBS-TPO· 2 0 g polyvinylpyrrolidone (mol wt 40,000), 0 2 g bovine serum albumin (BSA), make up to 100 mL with PBS-T. Make up fresh each day
5 Chloroform isoamyl alcohol (IAA)· (24.1)
6 1.25 m*M* dNTPs
7 1% Sodium dodecyl sulfate (SDS), 1 m*M* ethylenediamine tetra-acetic acid (EDTA), 10 m*M* Tris-HCl, pH 7.5
8. 3*M* NaAc

### 2.2. Chemicals

1. Protein A
2. Mineral oil
3 Absolute ethanol
4. RNase inhibitor (RNasin, Promega, Southampton, UK).
5 Superscript reverse transcriptase + buffer (Gibco-BRL, Paisley, UK)
6 *Taq* polymerase + buffer
7 Forward and reverse primers (*see* **Note 4**)

### 2.3. Equipment

1 Microcentrifuge
2 Water baths.
3 Micropipets
4. –20°C Freezer.
5 Microtiter plates (*see* **Note 2**)
6 PCR machine

## 3. Methods

### 3.1. Immune Capture (see Note 1)

1 Coat microtiter plates with 2 μg/mL protein A in coating buffer for 3 h at 37°C, using 200 μL per well. Wash plates three times for 30 s in PBS-T
2. Add antibody (*see* **Note 3**) diluted in PBS-TPO, using 200 μL per well, and incubate for 1 h at 37°C. Wash plates three times for 30 s in PBS-T.
3 Grind 0.5 g leaf tissue of superinfected plant and of control plants in 300 μL PBS-TPO.
4. Add virus or plant samples to well, diluted if necessary in PBS-TPO, using 150 μL per well, and incubate overnight at 4°C. Wash plates three times for 30 s in PBS-T
5 After final wash, extract viral RNA from plate with 1% SDS, 1 m*M* EDTA in 10 m*M* Tris-HCl, pH 7.5. Use 200 μL per well and incubate at 50°C for 5 min
6. Immediately transfer the samples to Eppendorf tubes containing 100 μL each of phenol and chloroform·IAA. Vortex for 5 min.
7 Centrifuge at 12,000*g* in a microcentrifuge for 15 min and recover the aqueous phase

8 Add 200 µL chloroform IAA to the aqueous phase from **step 6**, vortex for 5 min, and recentrifuge.
9 Recover aqueous phase again and precipitate the RNA by adding 20 µL 3*M* NaAc and 500 µL 100% cold ethanol Leave at –20°C overnight

### 3.2. Reverse Transcription

1. Pellet the RNA from **Subheading 3.1., step 9** by centrifuging at 14,000 rpm in a microcentrifuge for 15 min at 4°C. Dry pellet briefly and resuspend in 15 µL $H_2O$
2 Use a 5-µL aliquot of the resuspended RNA in the following mixture. 5 µL RNA, 5 5 µL $H_2O$, 1 µL RNase inhibitor, 1 µL reverse primer
3 Incubate at 65°C for 15 min and then on ice for 5 min
4 To each tube, add the following. 4 µL 5X superscript buffer, 1 µL 1 25 m*M* dNTPs; 2 µL 0 1*M* DTT; 0 5 µL superscript RT
5 Incubate at 42°C for 1 h and then at 65°C for 10 min

### 3.3. PCR

1 Use a 5-µL aliquot of first-strand synthesis (reaction from **Subheading 3.2., step 5**) in the following 5 µL first-strand synthesis, 10 µL 10X *Taq* buffer, 16 µL 1 25 m*M* dNTPs, 5 µL forward primer; 5 µL reverse primer, 58 5 µL H2O, 0 5 µL *Taq* polymerase
2 Overlay with 50–100 µL mineral oil
3 PCR cycle (*see* **Note 4**)

## 4. Notes

1 Controls It is most important to have the following negative controls to check for nonspecific binding of the superinfecting virus extract from transgenic plant not superinfected, extract from nontransgenic plant infected with the superinfecting virus. If nonspecific binding is found, parameters such as microtiter plates and blocking agents will need to be examined (*see* **ref. *29***) Also, a positive control of superinfecting viral RNA is required for the PCR in **Subheading 3.3.**
2 Microtiter plates Nonspecific reactions vary according to source of plate We found rigid Falcon plates to be the most satisfactory It is most important to fill the wells in the plate to the top in **Subheading 3.1., steps 1** and **2**, to prevent nonspecific binding in later stages.
3 This test is very sensitive and will easily detect any crossreactions between the antiserum and the CP of the superinfecting virus Initial tests should be performed to determine if this will be a problem If so, the antiserum can be preadsorbed with the superinfecting virus CP, preferably expressed in *Escherichia coli*, so that the superinfecting RNA does not contaminate the serum
4 The PCR conditions will depend on factors such as the composition of the primers and size of fragment being amplified. Details on primer selection and conditions for PCR can be found in textbooks on PCR (*see also* Chapter 48)

## References


1 Beachy, R. N (1988) Genetic engineering for virus protection in plants current results and future prospects, in *Research Bottlenecks for Commercialization and Beyond* (Mabry, T. J , ed ), University of Texas, Austin, TX, pp 39–47

2 Baulcombe, D (1989) Strategies for virus resistance in plants. *Trends Genet* **5,** 56–60

3 van den Wizen, P J M , Huisman, M J , Willink, D P-L., Jongedijk, E , Hoekema, A , and Cornelissen, B J C (1989) Engineering virus resistance in agricultural crops *Plant Mol Biol* **13,** 337–346

4 Gadani, F., Mansky, L M , Medici, R , Miller W A., and Hill, J H (1990) Genetic engineering of plants for virus resistance. *Arch Virol* **115,** 1–21

5 Hull, R (1990a) Non-conventional resistance to viruses in plants concepts and risks, in *Gene Manipulation and Plant Improvement II* (Gustafson, J. P., ed ), Stadler Genetics Symposium, Plenum, New York, pp 289–303.

6 Hull, R (1990b) Virus resistant plants: potential and risks *Chem Industry* **17,** 543–546

7 Hull, R (1994) Resistance to plant viruses obtaining genes by non-conventional approaches. *Euphytica* **75,** 195–205

8. Loesch-Fries, L. S (1990) Transgenic plants resistant to viruses, in *New Directions in Biological Control* (Baker, R and Dunn, P., eds ), UCLA Symposia on Molecular and Cellular Biology, New Series 112. Alan Liss, New York, pp. 629–639

9. Mayo, M A. and Barker, H (1990) Induction of resistance to plant viruses by transformation with virus genes. the poacher turned garnekeeper *Aspects Appl Biol* **24,** 65–77

10. Joshi, R L and Joshi, V (1991) Strategies for expression of foreign genes in plants. Potential use of engineered plants *FEBS Lett* **281,** 1–8.

11 Harms, C T (1992) Engineering genetic disease resistance into crops· biotechnological approaches to crop protection. *Crop Prot* **11,** 291–306.

12 Hull, R. and Davies, J W (1992) Approaches to nonconventional control of plant virus diseases. *Crit Rev Plant Sci* **11,** 17–33

13 Wilson, T M A (1993) New strategies to protect crop plants against viruses· pathogen-derived resistance blossoms *Proc Natl Acad Sci USA* **90,** 3134–3141

14 Fitchen. J H and Beachy, R N (1993) Genetically engineered protection against viruses in transgenic plants *Annu Rev Microbiol* **47,** 739–763.

15 Beachy, R. N (ed ) (1993) Transgenic resistance to plant viruses *Semin Virol* **4,** 327–416

16. Wilson, T M. A. and Davies, J. W. (1994) New roads to crop protection against viruses. *Outlook Agriculture* **23,** 33–39

17. de Zoeten, G. A. (1991) Risk assessment· do we let history repeat itself? *Phytopathology* **81,** 585,586

18 Tepfer, M (1993) Viral genes and transgenic plants *Biotechnology* **11,** 1125–1132

19 Vance, V. B., Berger, P H., Carrington, J C., Hunt, A. G , and Shi, X M (1995) 5' proximal potyviral sequences mediate potato virus X/potyviral synergistic disease in transgenic tobacco *Virology* **206,** 583–590

20. Lai, M M C (1992) RNA recombination in animal and plant viruses *Microbiol Rev* **56,** 61–79
21. Simon, A. E and Bujarski, J. J (1994) RNA–RNA recombination and evolution in virus-infected plants. *Annu Rev Phytopathol* **32,** 337–362
22 Goldbach, R and Wellink, J (1988) Evolution of plus-strand RNA viruses *Intervirology* **29,** 260–267
23 Strauss, E G and Strauss, J H (1991) RNA viruses genome structure and evolution *Curr Opin Genet Dev* **1,** 485–493
24 Greene, A E and Allison, R F (1994) Recombination between viral RNA and transgenic plant transcripts *Science* **263,** 1423–1425.
25 Mayo, M A and Jolly, C A. (1991) The 5'-terminal sequence of potato leafroll virus RNA· evidence of recombination between virus and host RNA. *J Gen Virol* **72,** 2591–2595
26 Sano, Y., van der Vlugt, R , de Haan, P., Takahashi. A , Kawakami, M., Goldbach, R , and Kojima, M. (1992) On the variability of the 3' terminal sequence of the turnip mosaic virus genome *Arch Virol* **126,** 231–238
27 Farinelli, L , Malnoe, P , and Collet, G. F. (1992) Heterologous encapsidation of potato virus Y strain O (PVY) with transgenic coat protein of PVY strain N ($PVY^N$) in *Solanum tuberosum cv* Bintje *Biotechnology* **10,** 1020–1025
28 Lecoq, H , Ravelonandro, M , Wopf-Scheibel, C , Monsion, M., Raccah B , and Dunez, J. (1993) Aphid transmission of a non-aphid-transmissible strain of zucchini yellow mosaic potyvirus from transgenic plants expressing the capsid protein of plum pox potyvirus. *Mol Plant–Microbe Interact* **6,** 403–406
29 Candelier-Harvey, P and Hull, R (1993) Cucumber mosaic virus genome is encapsidated in alfalfa mosaic virus coat protein expressed in transgenic plants *Transgenic Res* **2,** 277–285
30. White, S E , Habera, L F , and Wessler, S R (1994) Retrotransposons in the flanking regions of normal plant genes a role for *copia*-like elements in the evolution of gene structure and expression *Proc Natl Acad Sci USA* **91,** 11,792–11,796
31 Flavell, A. J., Dunbar, E., Anderson, R., Pearce, S. R , Hartley, R., and Kumar, A (1992) *Tyl-copia* group retrotransposons are ubiquitous and heterogeneous in higher plants *Nucleic Acids Res* **20,** 3639–3644.
32. Voytas, D F , Cummings, M P , Konieczny, A , Asubel, F. M , and Rodermel, S R (1992) *Copia*-like retrotransposons are ubiquitous among plants. *Proc Natl Acad Sci USA* **89,** 7124–7128.
33 Hirochika, H and Hirochika, R. (1993) *Tyl-copia* group retrotransposons as ubiquitous components of plant genomes *Jpn J Genet* **68,** 35–46
34. Johns, M. A , Mottinger, J., and Freeling, M. (1985) A low copy number, copia-like transposon from maize. *EMBO J* **4,** 1093–1102.
35. Grandbastien, M -A , Spielmann, A., and Caboche, M. (1989) Tntl, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics *Nature* (London) **337,** 376–380

36. Varagona, M. J , Purugganan, M. D., and Wessler, S. R (1992) Alternative splicing induced by insertion of retrotransposons into the maize *waxy* gene *Plant Cell* **4,** 811–820
37 Hirochika, H. (1993) Activation of tobacco retrotransposons during tissue culture *EMBO J* **12,** 2521–2528
38 Harrison, B. D and Robinson, D. J (1988) Molecular variations in vector-borne plant viruses epidemiological significance *Phil Trans R Soc London B* **321,** 447–462
39 Atreya, C D , Raccah, B , and Pirone, T P (1990) A point mutation in the coat protein abolishes the aphid transmissibility of a potyvirus *Virology* **178,** 161–165.
40. Atreya, P L., Atreya, C D., and Pirone, T. P. (1991) Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus *Proc Natl Acad Sci USA* **88,** 7887–7891.
41 Lindbo, J A and Dougherty, W G (1992) Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts *Virology* **189,** 725–733.

# 55

# Potential Benefits of the Transgenic Control of Plant Viruses in the United Kingdom

**Ian Barker, Christine M. Henry, Miles R. Thomas, and Rebecca Stratford**

## 1. Introduction

Much has been written on the possible risks arising from the use of virus-resistant transgenic crop plants but little of the benefits that might result. Many of the potential benefits are self-evident and relate to improved disease control, but others are less so and arise from such indirect effects as reductions in insecticide usage for the control of insect transmitted viruses. This paper attempts to discuss and (where possible) quantify possible benefits from transgenic approaches in relation to UK crop protection, but is in nature speculative as, to date, no such crops are in commercial production in the United Kingdom.

## 2. Benefits

### *2.1. Agronomic*

One of the limiting factors in breeding for virus resistance is the availability of suitable resistant germplasm. Ideally, resistant germplasm consists of a single monogenetically inherited dominant resistance gene, which is present in a related variety or close relative. Polygenic or multigenic resistances are difficult to utilize, since several or many genes are required to confer resistance, and the probability of combining these genes in one line, together with other desirable traits, is very low. If the resistance is present only in related species rather than in existing cultivars, expensive backcrossing to the cultivated parent is required to remove the alien germplasm. Linkage to undesirable traits can be difficult to break, since the recombination is less frequent between distantly related sequences.

From *Methods in Molecular Biology, Vol 81 Plant Virology Protocols*
*From Virus Isolation to Transgenic Resistance*
Edited by G D Foster and S C Taylor 

The ability to introduce virus resistance into susceptible varieties or advanced breeding lines without affecting the intrinsic properties of that cultivar is a key feature that distinguishes transgenic virus resistance from conventional breeding strategies. Breeding relies on hybridization of two parental genotypes, followed by selection of progeny with the desired set of characters with the consequence that many lines are not developed through to commercial cultivars owing to a lack of one or more traits. The ability to incorporate virus resistance into finished varieties has the advantage of enabling the breeder to place greater emphasis on the selection of other more complex traits during the breeding process

In addition, transgenic resistance provides an alternative source of virus resistance, which is of particular utility when host resistance is either unavailable or only present in a form that is difficult to access. The diversity of strategies for transgenic resistance and the wide range of host–virus combinations for which resistance has been reported suggests that most virus diseases could potentially be controlled by this approach. The level of resistance conferred by transgenic strategies has been reported to be similar to that conferred by host resistance genes, and field trial reports indicate that the resistance is of agronomic value. Once resistance has been introduced into a cultivar, that cultivar can be used in conventional breeding programs to distribute the virus resistance more widely.

### 2.2. Environmental

The principal benefit to the environment that would arise from the adoption of virus-resistant transgenic crops would be the reduction in the usage of pesticides, applied for the control of arthropod and nematode virus vectors. This in turn would reduce the amount of damage caused to nontarget organisms in the agricultural and nonagricultural environment. Higher levels of pests, such as aphids, could then also be tolerated on crops that would in turn increase the base of the food chain for other organisms, such as farmland birds. There has been concern that the documented decline in the populations of many farmland birds, such as the gray partridge *(1)* and others *(2)*, have been partly brought about by the widespread use of broad spectrum insecticides. Many of the nontarget organisms, such as insect predators like carabid beetles, are also beneficial organisms; and any reduction in damage to these populations (by the reduction of the use of broad spectrum insecticides) could have a consequential effect of further reductions in pesticide use for the control of their prey species.

Additional benefits might include the extension of the use of biological control programs for pests of protected crops in which one problematic pest in a series of pests can only be controlled by chemical means, thereby limiting the

**Table 1**
**Usage of Insecticides for the Control of Aphid Vectors of Virus Diseases of Selected Arable Crops in 1992 (Principal Usage)**

| Crop | Disease | Area treated, ha | Amount applied, tons a i |
|---|---|---|---|
| Sugar beet | Virus yellows[a] | 196,346[b] | 27 1 |
| Wheat | Barley yellow dwarf | 1,604,919[c] | 127.46 |
| Winter barley | Barley yellow dwarf | 539,544[c] | 11.68 |
| Oilseed rape | Beet western yellows | 15,045[d] | 0 88 |
| Ware potatoes | Potato viruses[e] | 192,113 | 24.01 |
| Seed potatoes | Potato viruses[e] | 69,137 | 9 07 |
| Total | All viruses | 2,617,104 | 199 32 |

[a]Caused by beet mild yellowing virus (syn beet western yellows virus) and beet yellows virus
[b]Excludes aldicarb but includes tefluthrin
[c]Excludes all seed treatments
[d]Includes only named insecticides where aphid control specified
[e]Principally potato leaf roll virus

uptake of IPM (integrated pest management) strategies. A good example of the latter would be the possible increase in the use of biological control, for aphid and other pests, in glasshouse crops if the thrips vectors of tomato-spotted wilt virus (TSWV) were made less important. Controlling the virus directly through transgenic, resistant crops rather than spraying to control the vector would be one such mechanism to achieve this end. It is also a stated aim of the ministry to rationalize pesticide usage where possible

A list of pesticide use on arable crops in the United Kingdom for 1992 *(3)*, which can be directly attributed to the control of insect vectors of important viral diseases, is shown in **Table 1**. The total treated area of arable crops in the United Kingdom receiving insecticide amounted to some 3,584,554 ha in 1992; arable crops account for 92% of all pesticide use in the UK. Hence the amount of insecticide specifically used to control plant viruses (**Table 1**) represents at least 67% of all insecticides used in United Kingdom agriculture (crop protection) and includes chemicals of great topical concern in relation to the environment. A similar pattern of use was seen in corresponding figures for insecticide use in the United Kingdom in 1994 *(4)*, although the percentage of use on arable crops, relating principally to the control of plant virus vectors, dropped to 42% (1,330,590 ha) of the total applied (3,114,885 ha).

A number of other plant virus diseases, particularly of horticultural crops, are controlled with insecticides and nematicides; but either their use is on a very small scale (compared to the use on arable crops) or virus control is not

the principal purpose. Thus cauliflower mosaic virus and turnip mosaic virus in vegetable brassicas are controlled to some extent by spraying for their aphid vectors using insecticides. However, the principal need for the chemical treatment would be to control the aphids themselves along with other pests, such as caterpillars. Thus the development of transgenic virus-resistant varieties might not result in a reduction in pesticide usage.

The extent to which the deployment of transgenic virus-resistant varieties would reduce these pesticide figures in the United Kingdom would depend mainly on economic factors and whether breeding companies perceived that a suitable market existed. It can, however, be seen that there is potential for very large-scale reductions in pesticide use, if suitable completely resistant transgenic varieties were made available and were widely adopted. Regulatory authorities should take note of this potential in the light of public and governmental concerns over pesticide usage; **Table 1** can be used to estimate the likely environmental benefits of a given virus-resistant crop being widely grown.

### 2.3. Public Health

In theory, public health benefits could accrue from a reduction in insecticide usage of those chemicals used to protect the crops listed in **Table 1** from the virus diseases mentioned, if transgenic resistant crops were widely grown.

However, there is no evidence (MAFF monitoring studies) of any detectable residues of these insecticides being present in the foodstuffs originating from these crops. This is principally owing to these insecticides being used as foliar sprays or as seed treatments early in the life of the crop, and either no subsequent movement of the products or residues to the edible parts of the plant or simply the breakdown of the product long before harvest. Processed products, such as beet sugar or rape-seed oil, are even less likely to contain the residues of insecticides applied to control aphid virus vectors. Residues of insecticides used to protect seed potatoes would also not be present in the tubers produced from progeny crops.

The only possible public health benefits might arise from a reduction in operator exposure by farm and allied workers in the manufacture, distribution, and application of insecticides used to control virus vectors. These benefits might be significant considering the very large amounts of insecticides involved and their known or suspected harmful effects *(5,6)* (both acute and long-term). Any actual benefits would be very difficult to quantify.

It is thus unlikely that any major public health benefits would follow from the widespread adoption of transgenic virus-resistant crops. A large reduction in the use of insecticides might however help to reduce the risks to health as perceived by the public, which is greater than can be supported by known facts *(5)*. It would appear that the principal benefits would be environmental and agronomic.

## 3. Case Studies

### *3.1. Viruses Transmitted by Soil Fungi*

The most important of these are the viruses transmitted by the Plasmodiophorales. These viruses are transmitted inside the zoospores and resting spores of fungi such as *Polymyxa betae*. The long-lived nature of the resting spores of these fungi makes any control by crop rotations impossible. There is no viable chemical method for their control and breeding for resistance by conventional means is unsatisfactory

There are two virus diseases of this type, which are of importance to UK agriculture, both discussed in detail below. These are the mosaic viruses of barley and rhizomania disease of sugar beet.

### *3.2. Barley Yellow Mosaic Virus and Barley Mild Mosaic Virus*

Both barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) cause a mosaic disease of winter barley in the UK *(7)*. Both viruses are transmitted by the weakly parasitic root infecting fungus, *Polymyxa graminis*, whose resting spores can survive in soil for at least 10 yr. The spread of the disease is by soil movement, wind, water, or cultivation practices. It has been estimated that 13% of barley fields were infected by one or more of these viruses in 1988, and the disease is still spreading. Yield losses due to these two viruses are not precisely known, but losses of up to 30–50% have been reported from heavily infected areas. This could equate to a financial loss of about £14 million p.a. if these areas were uniformly infected. Some reduction of the virus disease can be seen if crops are sown later, especially if the disease is due to BaMMV *(8)*. However, the planting of resistant varieties is the principal control method.

Conventional breeding for resistance to these viruses has resulted in the production of some resistant varieties of barley A recessive gene, *ym4*, identified from a common parent, the Yugoslavian spring barley land-race Ragusa, confers resistance to both BaYMV and BaMMV. *Ym4* has been bred into such feed varieties of barley as Target, Epic, and Willow; but it is beginning to be seen in malting varieties too. There is also another source of tolerance to BaYMV and BaMMV in varieties such as Augusta, Sonja, and Sprite; but the genetic basis of the tolerance is not understood

In the late 1980s, a resistance-breaking strain of BaYMV (BaYMV race 2) was found in Germany, France, and the United Kingdom that overcame the *ym4* resistance *(9–11)*. Twenty-five separate outbreaks of the resistance-breaking isolate have been reported to date from a wide geographical area. Breeders are therefore now searching for other sources of resistance. There is genetic diversity for BaYMV and BaMMV resistance in barley landraces and varieties

from the Far East that could be used to widen the genetic base for European varieties; but some of this is difficult to utilize, since it is inherited recessively and is oligogenic *(12)*. Breeding with these exotic germplasms is also complicated because they are agronomically unsuited to the United Kingdom Time-consuming backcross programs are needed to combine the resistance with an appropriate agronomic background. A transgenic approach to breeding would speed up these programs. It is particularly important at present to breed resistance to BaMMV into the malting varieties, and it may prove to be more feasible to do this by a transgenic than a conventional breeding program.

### 3.3. Rhizomania Disease of Sugar Beet

Rhizomania disease of sugar beet is caused by beet necrotic yellow vein virus (BNYVV), which is transmitted by the soil fungus, *Polymyxa betae*. The disease was first found in Italy in the 1950s but has since spread to most of the sugar-beet-growing areas of the world. The disease was first reported in the United Kingdom in 1987, and since then there have been an additional 76 outbreaks in the United Kingdom. Investigations of the yield effect of rhizomania in the United Kingdom have suggested that yield losses would be of the order of 53–75% or 3.6–4.9 tons of sugar per hectare if the disease continued to spread unchecked *(13)* This is in a crop with an annual farm gate value of about £300 million. The disease is currently restricted to certain areas in East Anglia; and the United Kingdom Ministry of Agriculture, Fisheries & Food (MAFF) pursue a statutory control policy to limit the spread of the disease. Control of the disease is possible by using methyl bromide soil sterilization *(14)*, but this is too expensive for routine use on a field scale and is being phased out under the Montreal Protocol. This leaves the production of resistant cultivars the only practicable solution to its long-term control

There are a limited number of sources of partial resistance to BNYVV in sugar beet itself; these have been used to produce some of the new sugar beet cultivars with partial resistance to the disease. Most of these cultivars have reduced sugar yield and root quality compared to susceptible cultivars. Also, they have undesirable agronomic attributes, such as increased bolting. Recently developed cultivars, such as Ballerina, are near to the acceptable quality for sugar beet cultivars with a 1–3% yield penalty and a reasonable level of resistance to the virus. These cultivars do however still become infected by BNYVV; and there are fears that they may build up inoculum levels in soils while masking disease spread, leading to widespread infection. The lack of immunity to the disease has recently posed a major problem for MAFF in that they are reluctant to deregulate the disease and allow partially resistant cultivars to be grown if there is any possibility that a breakdown of such resistance could take place in the near future. The development of a transgenic cultivar of

sugar beet with immunity to BNYVV would remove their concerns and allow infected land in East Anglia to be brought back into productive use. Also, the ability to move resistance genes into ideal agronomic backgrounds would speed up the breeding programme.

## 4. Insect Transmitted Viruses

### *4.1. Barley Yellow Dwarf Virus (BYDV)*

BYDV infects all the small-grain cereals, along with maize and the majority of important pasture grasses. It has a worldwide distribution and is one of the most important plant-disease agents in the world. Actual losses due to BYDV in the United Kingdom are not known; but yield losses of 30–40% have been measured in field trials (unsprayed plots) ***(15)***, and up to 90% of crops can be affected in some years ***(16)***. Actual average losses in wheat and barley are probably in the region of 0.5–5%, which would represent a loss of about £10–100 million p.a., with epidemic years occurring once or twice in a decade. Losses of up to 26% have been reported for various ryegrass species ***(17)***

BYDV belongs to the luteovirus group and is spread by aphid vectors in the persistent manner. There are 24 known species of aphid capable of transmitting BYDV; but only five species are thought to be significant vectors in the United Kingdom, with *R padi* and *S avenae* being the most important. At least three different strains of the virus occur in the United Kingdom, which differ in their vector specificity ***(18,19)***, virulence, and geographical distribution. The principal control measure for the virus in cereal crops in the United Kingdom is through the application of broad spectrum insecticides, particularly on winter cereals, though control using resistant or tolerant varieties is practiced outside Northwest Europe. BYDV is not usually controlled in pasture grasses in the United Kingdom, but some effort to breed more resistant varieties has been expended in the past ***(17)***. The epidemiology and control of BYDV in Britain has been reviewed by Plumb ***(20)***.

Progress in breeding for tolerance to BYDV in barley has been made using the *Yd2* gene, first identified in Ethiopian barley lines ***(21)***. The *Yd2* gene is now present in the new United Kingdom spring barley cultivar Amber Older varieties, such as Coracle and Vixen, are also resistant to BYDV but are not grown widely since they are inferior to current cultivars and are particularly susceptible to *Rhynchosporium secalis*. Selection of resistant varieties is difficult owing to the lack of reproducible infection and variation in symptom expression. In addition to major genes, there are also nonspecific minor genes that have been accumulated in barley cultivars through many years of selection, which confer some tolerance to infection.

The wheat crop is attacked by a number of viruses, of which BYDV is the most important in the United Kingdom. However, wheat is generally not as

sensitive as barley to the effects of BYDV infection. There are no examples of immunity or resistance to BYDV in wheat, although the North American bread-wheat cultivar Anza and several CIMMYT (International Maize and Wheat Improvement Centre, Mexico) bread-wheats show partial resistance to infection with BYDV. Most United Kingdom varieties are susceptible to infection, although the degree of susceptibility varies considerably.

New sources of resistance have been identified in a wide range of Triticeae accessions, in particular the genera *Hordeum*, *Leymus*, *Elymus*, and *Elytrigia* ***(22,23)***; and attempts are also being made to introduce the major gene *Yd2* from barley into a wheat background ***(24)***.

A transgenic approach to the control of BYDV in UK cereals would have to compete with current chemical control, which is both cheap and effective, but would have clear environmental advantages. Wheat would appear to represent a better candidate for transformation for virus resistance than barley because of the lack of suitable major gene resistance in the former.

### 4.2. Potato Viruses

The most important viruses of potato are potato leaf roll virus (PLRV), potato virus Y (PVY), and potato virus X (PVX). There are several known sources of resistance to these viruses among the cultivated and wild potato species, and breeders have incorporated many of them into new potato crops.

PVX and PVY can be controlled by dominant genes conferring either hypersensitivity (Nx, Ny) or extreme resistance (Rx, Ry). The provenance of these genes is well-characterized ***(25)***, although genes from some sources are more effective than others. They are relatively easy to transfer to new varieties in breeding programs, since they are monogenic, and selection for resistance to PVX and PVY can be achieved via mechanical techniques. In contrast, breeding for PLRV resistance has met with only limited success due to complex genetic control and also by the difficulty in screening, since PLRV is transmitted by aphids. Sources of partial resistance include *S. acaule*, *S dernissum*, *S chacoence*, and *S. tuberosum* subsp *andigena*. Some selections of *S brevidans* are reported as having extreme resistance to PLRV, but the resistance is again most likely to be oligogenically controlled ***(26)***.

Overall, approx 40% of potato varieties on the NIAB 1995 recommended list have good resistance to either PVX or PVY. However, combining resistance into any one particular cultivar has proved difficult, and only one variety (Sante) on the current list has good resistance to all three major viruses. The potato industry displays very slow uptake of new varieties and so the varieties grown most widely are often susceptible to virus infection. However, this is not necessarily problematic in countries with good virus-free seed-growing areas and certification schemes. Transgenic approaches for improving potato

virus control might then be most appropriate for PLRV but may also offer potential for combining resistances to separate potato viruses.

## 5. Conclusions

In summary, transgenic virus resistance broadens the range of resistant germplasm and can confer resistance to viruses that have previously been difficult to control using host resistance. It also increases the diversity of control mechanisms: the combination of transgenic and host resistances may confer a greater level of resistance and possibly increase the durability of the resistance. Furthermore, the ability to transform crop species directly should lead to an increase in the rate at which virus-resistant cultivars with good agronomic and quality traits are developed.

The rate of uptake of the technology in the United Kingdom will depend largely on economic factors as well as public and industry acceptance. A better appreciation of the potential benefits that might arise from growing virus-resistant transgenic crops should help to promote the technology, in general, particularly in relation to public acceptance

## References

1 Campbell, L H and Cook A S (eds ) (1997) *Indirect Effects of Pesticides on Birds*. Joint Nature Conservation Council, Peterborough, UK
2 Potts G. R. (1986) *The Partridge Pesticides, Predation and Conservation* Collins, London, UK
3 *Pesticide Usage Survey Report 108 Arable Farm Crops in Great Britain* (1992) M.A F.F., London, UK
4. *Pesticide Usage Survey Report 127 Arable Farm Crops in Great Britain* (1994) M.A F.F., London, UK
5. *The BMA Guide to Pesticides, Chemicals and Health* (1992) British Medical Association. Edward Arnold.
6. Hallenbeck, W. H and Cunningham-Burns, K M (1985) *Pesticides and Human Health*. Springer Verlag.
7 Adams, M J and Hill, S A. (1992) *Soil-Borne Viruses of Cereals The UK Situation* Home Grown Cereal Authority Leaflet.
8. Adams, M J., Overthrow, R , and Carver, M F. F. (1996) Effects of cultivar and sowing date on the incidence of barley mosaic viruses and on yield *Proc of the Third Symposium of the International Group on Plant Viruses with fun gal vectors* (Sherwood, J. L. and Rush, C M , eds ), Dundee, American Society of Sugar Beet Technologists, Denver, CO.
9. Huth, W. (1989) Management of yellow mosaic inducing viruses on barley by selection of resistant cultivars. *Bulletin OEPP/EPPO Bulletin* **19,** 547–553
10 Adams, M J (1991) The distribution of barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BMMV) in UK winter barley samples 1987–1990 *Plant Pathol* **40,** 53–58

11 Harori, D J , Fouchard, M , and Lapierre, H. (1990) Resistance to barley yellow mosaic virus and barley mild mosaic virus in barley *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Braunschweig*, pp 109–112
12. Ordon, F. and Friedt, W (1993) Mode of inheritance and genetic diversity of BaMMV resistance of exotic barley germplasms carrying genes different from 'ym4'. *Theoretical Appl Genet.* **86,** 229–233
13. Henry, C. M. (1996) Rhizomania—its effect on sugar beet in the UK. *Brit Sugar Beet Rev* **69,** 24–26
14. Henry, C M., Bell, G. J , and Hill, S A. (1992) The effect of methyl bromide fumigation on rhizomania inoculum in the field. *Plant Pathol* **41,** 483–489
15 Dawson, G W , Griffiths, D C , Hassanali, A., Pickett, J A., Plumb, R. T , Pye, B. J., Smart, L. W , and Woodcock, C M (1986) Antifeedants a new concept for control of BYDV in winter cereals. *Proceedings 1986 British Crop Protection Council Conference—Pests and Diseases* **3,** 1001–1008
16 King, J. E (1977) The incidence and economic significance of diseases in England and Wales. *Proceedings: 1977 British Crop Protection Council Conference—Pests and Diseases* **3,** 677–687
17 Catherall, P L and Parry, A. L (1987) Effects of barley yellow dwarf virus on some varieties of Italian, hybrid and perennial ryegrasses and their implication for grass breeders *Plant Pathol* **36,** 148–153
18. Barker, I (1990) Barley yellow dwarf in Britain, in *World Perspectives on Barley Yellow Dwarf Virus* (Burnett, P., ed ), CIMMYT, Mexico, pp 39–41
19 Holmes, S J (1985) Barley yellow dwarf virus in ryegrass and its detection by ELISA *Plant Pathol* **34,** 214–220.
20. Plumb, R.T. (1986) A rational approach to the control of barley yellow dwarf virus *J Royal Agri Soc Engl* **147,** 162–171.
21 Schaller, C W , Qualset, C O., and Rutger, J N (1964) Inheritance and linkage of the Yd2 gene conditioning resistance to the barley yellow dwarf virus in barley *Crop Science* **4,** 544–548
22. Comeau, A. and Plourde, A (1987) Cell tissue culture and intergenic hybridisation for barley yellow dwarf virus in wheat. *Can J Plant Pathol* **9,** 188–192
23 Xu, S J , Banks, P M , Dong, Y S , Zhou, R H , and Larkin, P. J. I (1994) Evaluation of Chinese Triticae for resistance to barley yellow dwarf virus (BYDV) *Genet Res Crop Evolution* **41,** 35–41
24 McGuire, P E. (1984) Status of an attempt to transfer the barley yellow dwarf virus resistance gene Yd2 of barley to hexaploid wheat, in *Barley Yellow Dwarf* (Burnett, P ed.), CIMMYT, Mexico, pp 113–119
25 Valkonen, J P T. (1994) Natural genes and mechanisms for resistance to viruses in cultivated and wild potato species (*Solanum* spp ) *Plant Breeding* **112,** 1–15
26 Jones, R. A C (1979) Resistance to potato leaf roll virus in *Solanum brevidens Potato Res* **22,** 149–152.