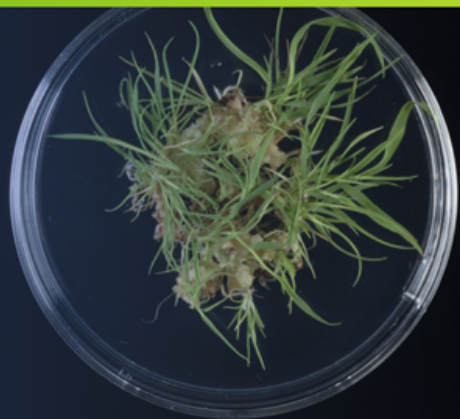


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Jim M. Dunwell
Andy C. Wetten *Editors*

Transgenic Plants

Methods and Protocols

Second Edition

 Humana Press

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Second Edition

Edited by

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Preface

In 2010, the global area of transgenic crops reached 148 million hectares, an 87-fold increase since 1996, the first year of planting. This makes such technology the most rapidly adopted one in the history of modern agriculture.

This success has been based on the scientific foundation developed in many international laboratories since the 1980s, and it is the purpose of the present volume to provide access to the continuous improvements being made for the production and analysis of transgenic plants. Importantly, this volume is designed to complement and extend the information published in this same series *Transgenic Plants: Methods and Protocols* in 2005.

The present collection of novel methods is divided into eight parts which cover a range of protocols for both model and crop species. The first part covers various methods for the selection and detection of transgenic plants, and this is followed by a series of sections that describe specific methods for algae and then for higher plants. These latter sections are divided into those devoted to monocots, with an emphasis on rice, and then to dicots such as plum, grape, and cotton. Part VI covers a rapidly growing area of research, namely, the specific targeting and directed silencing of plant genes. Many of these technologies will cause considerable debate among the various regulatory regimes as they are not always detectable by molecular methodology and therefore are indistinguishable from mutations produced either spontaneously or by other methods. Part VII describes a range of applied examples to generate plants with modified metabolism or the production of pharmaceuticals. Finally, Part VIII describes the experience gained from the growth of transgenic *Arabidopsis* in the field.

Throughout the preparation of this volume, stimulated by the enthusiasm of the series editor John Walker, we have been aided by the continual high quality input from the large number of authors and we thank them all for their respective contributions.

Reading, UK

*Jim M. Dunwell
Andy C. Wetten*

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Part I

Selection and Detection Methods

Chapter 1

Employment of Cytokinin Vectors for Marker-Free and Backbone-Free Transformation

Craig M. Richael and Caius M. Rommens

Abstract

Marker-free methods of plant transformation sacrifice the advantages of a selectable marker during regeneration or add work after regeneration to remove the marker. On the positive side, there is no stably integrated marker gene in the plant genome to present regulatory hurdles or potential biosafety hazards once the plant is released to the environment. A marker-free method that is simple and adaptable to multiple crop species—even asexually propagated species—is presented herein. This method employs an engineered vector that utilizes the isopentenyltransferase (*ipt*) to drive the regeneration of intragenic cells containing the gene(s) of interest. The *ipt* gene also acts as a marker to screen against events where the vector backbone is stably integrated.

Key words: Cytokinin, Isopentenyltransferase, Intragenic, Cisgenic, Transgenic, Marker-free, *Agrobacterium*, Potato, Tomato

1. Introduction

At about the turn of the century, intragenic or cisgenic crops were proposed as an alternative to the currently released transgenic crops (1, 2). Unlike transgenics, intragenic crop plants harbor no foreign DNA; all introduced DNA sequences, including gene and gene regulatory elements, are of plant origin and derived from sexually compatible species. Moreover, no extraneous vector DNA (vector backbone) or foreign selection marker genes are permissible for co-transfer. Using intragenesis, one can manipulate the expression of native genes without providing the target plant with a new trait that neither occurs in the recipient species in nature nor can be introduced through traditional breeding. Intragenesis most closely approximates traditional breeding in that no foreign DNA is involved. The method is employed to correct deficits of elite

cultivars derived by traditional breeding, particularly for crops consumed as food by humans (3, 4). By altering the expression on one or a few genes, a cultivar can be improved without the disadvantage of genetic drag associated with a sexual cross.

Transgenes are novel genes to a plant that essentially broaden the plant's gene pool. It is possible that the introduced gene grants an adaptive advantage that could flow to either unmodified crop plants of the same species or wild relatives. This potential safety issue caused the development of a stringent regulatory process that prevents the release of the genetically modified plant until risks are fully assessed. In contrast, the intragenic approach may require less regulatory oversight, provided that the plant product is substantially equivalent to the untransformed parent except for the incorporated trait (5).

For intragenesis to succeed, marker-free selection methods must be developed and optimized. Antibiotic or herbicide resistance genes of bacterial origin can no longer be used to identify transformed cells and select for genetically modified events. Given the prevalence of vector backbone sequences in *Agrobacterium*-based transformation events (6–9), it would also be useful to include in the marker-free method a way to screen against backbone integration events during regeneration of the transformed cells. The current method explains the use of an engineered vector that contains the gene(s) of interest, drives the regeneration of intragenic cells, and provides a marker to screen against stably integrated backbone fragments. Presented are the methods for crop plants for which we have successfully applied the technology. If traditional regeneration protocols for a particular crop typically employ cytokinins, we expect this method to be applicable.

2. Materials

1. *Agrobacterium* strain LBA4404 or C58C1 transformed with gene of interest (e.g., neomycin phosphotransferase) within the T-DNA and the isopentenyltransferase gene engineered within the vector backbone. A vector suited to the generation of marker-free, backbone-free plants is described previously (2).
2. Timentin (or ticarcillin) stock (RPI, Prospect, IL) dissolved in deionized water at 100 mg/mL concentration. Store at -20°C .
3. Luria (Miller's) broth medium, 25 g/L (ISC BioExpress, Kaysville, UT).
4. Propagation medium for potato; 0.5× M516 medium (PhytoTechnology Laboratories, Shawnee, KS) dissolved in deionized water according to manufacturer's instructions

supplemented with 3% sucrose and 2 g/L gelzan (Caisson Labs, Inc., N. Logan, UT), pH 5.7.

5. Propagation medium for canola. 0.5× M404 (PhytoTechnology) dissolved in deionized water according to manufacturer's instructions supplemented with 3% sucrose and 2 g/L gelzan.
6. MS liquid medium. 1× M404 dissolved in deionized water according to manufacturer's instructions supplemented with 3% sucrose.
7. Germination medium for tomato. 1× M404 medium containing 1.5% sucrose and 2 g/L gelzan.
8. Co-culture medium. 0.1× M404 dissolved in deionized water according to manufacturer's instructions supplemented with 3% sucrose and 6 g/L plant tissue grade agar, pH 5.7.
9. Regeneration medium. Murashige and Skoog modified basal medium with Gamborg vitamins (M404, PhytoTechnology Laboratories) dissolved in deionized water according to manufacturer's instructions supplemented with 3% sucrose and solidified with agar, pH 5.7.
10. Growth chamber and growth conditions. Percival growth chambers (models CU36L4 or L5, Percival Scientific, Inc., Perry, Iowa, USA) are programmed at a constant 24°C and a light/dark duration of 16/8 h. Light intensity in the Percival models ranges from 140 in the L4 model to 155 $\mu\text{mol}/\text{m}^2/\text{s}$ in the L5 model.

3. Methods

Precise execution of the methods described will provide a simple means to create marker-free and backbone-free plants. See Table 1 for expected transformation frequencies using these methods for the crop varieties tested. Myriad problems can arise when applying a transformation procedure to a new crop species or a variety within a crop species. Many crops are recalcitrant to transformation and regeneration protocols. Some cultivars may readily transform and regenerate, but another cultivar of the same crop species will not. We expect that the same problems will be encountered when applying the *ipt*-based method to crops or cultivars not covered by our work. We expect that if a regeneration protocol calls for cytokinin, the *ipt*-based method will work. Optimizations of the method begin with variety and explant selection and could include medium amendments and promoter selection for the *ipt* gene. It has been observed that the longer and more complex constructs result in slower regeneration and lower transformation frequencies.

Table 1
***ipt*-based regeneration and transformation rates of various crop cultivars.**
***Agrobacterium* strain LBA4404 harboring a vector with a neomycin**
phosphotransferase II cassette within the borders and an *ipt* cassette driven
by the ubiquitin-3 promoter (Ubi7 promoter for canola) in the vector backbone
was used for all experiments. Frequencies represent an average $\pm$ standard error
of three independent experiments

Species, variety	Explant	Frequency (%)		
		Regeneration ^a	Transformation ^b	Backbone-free ^c
Potato, Ranger Russet	Internode	90.6 + 1.2	7.8 $\pm$ 0.6	5.8 $\pm$ 1.0
Potato, Bintje	Internode	92.3 + 3.8	5.4 $\pm$ 0.7	4.1 $\pm$ 0.4
Potato, Russet Burbank	Internode	83.3 + 2.4	2.9 $\pm$ 0.9	2.7 $\pm$ 0.7
Potato, Atlantic	Internode	73.6 + 2.7	4.9 $\pm$ 0.8	3.8 $\pm$ 0.3
Tomato, Moneymaker	Hypocotyls	19.8 + 5.5	5.4 $\pm$ 0.3	3.9 $\pm$ 0.7
Tobacco, Petit Havana	Leaf disc	100	13.5 $\pm$ 2.1	9.9 $\pm$ 0.4
Canola, Westar	Internode	31.7 + 0.6	3.5 $\pm$ 0.7	3.1 $\pm$ 0.2

^aNumber of explants forming a shoot/total number of explants transformed
^bBased on PCR screening for the *npt II* gene or gene of interest in all normal-looking shoots. Only one normal-looking shoot removed from each explant providing one
^cAs determined by PCR screening for the backbone fragment DNA

3.1. Explant
Co-cultivation with
Agrobacterium

1. Grow the *Agrobacterium* harboring the vector of interest overnight at 28°C in LB medium containing antibiotics to select for bacteria and vector (see Note 1).
2. Grow tenfold dilutions of the overnight cultures for 5–6 h to log phase and precipitate at 3,000 rpm (1962×g).
3. Wash the pellet in M404 liquid medium and resuspend in the same liquid medium to obtain a cell density of 0.2 (OD₆₀₀).
4. Incubate the explants with bacteria for 10 min, blot on sterile filter paper, and transfer to co-cultivation medium. As a control, introduce a subset of explants to *Agrobacterium* harboring a vector without the *ipt* gene. These explants should not regenerate on hormone-free regeneration medium.

3.2. Potato
Transformation

1. Maintain stock plants from which explant material is taken in Magenta boxes with 40 mL of potato propagation medium.
2. Cut internode segments of 4–6 mm from 4-week-old plants.
3. Infect with *Agrobacterium* and transfer to co-cultivation medium.

4. After 2 days co-cultivation, transfer the explants to a new plate containing regeneration medium with 150 mg/L timentin to eliminate *Agrobacterium* (see Note 2).

3.3. Tomato Transformation

1. Surface sterilize seeds of tomato (cv. Moneymaker) in 10–20% bleach and 0.01% Tween 20 for 20 min and rinse with sterile water three times.
2. Germinate the seeds on tomato germination medium.
3. Cut hypocotyls of 12- to 14-day-old seedlings into segments of 5–8 mm.
4. Co-cultivate *Agrobacterium* cultures with the hypocotyl segments for 10 min then transfer to co-culture medium for 2 days.
5. Shoots regenerate on regeneration medium that is refreshed every 2 weeks (see Note 3).

3.4. Tobacco Transformation

1. Carry out *ipt*-based transformation as described for potato except that leaf segments of 4-week-old in vitro grown plants are used as explant material.

3.5. Canola Transformation

1. For *ipt*-based transformation, use internode segments of canola (cv. Westar) as explants. No success was derived with cotyledon petiole explants of this cultivar.
2. Cut internode segments (~5 mm) from 4- to 6-week-old canola plants grown in vitro on canola propagation medium.
3. Carry out transformation and subsequent regeneration as described for potato.

3.6. Phenotyping and Genotyping Transformed Plants

1. Depending on the crop species and growing conditions, shoots appear after 1–2 months postinfection (see Note 4).
2. No shoots arise from explants not infected with the *ipt* vector placed on regeneration medium. Explants provide a mix of normal- and abnormal-looking shoots. The abnormal shoots tend to be chlorotic, highly branched, and stunted (see Fig. 1). Their leaves are smaller than normal (see Note 5).
3. The abnormal shoots root poorly or not at all once excised from the explants. Ignore these shooty, irregular plantlets as these contain stably integrated *ipt* and, hence, vector backbone.
4. Excise only a single normal-looking shoot from each explant to ensure that no duplication of a single transformation event occurs. Not all explants provide a normal-looking shoot.
5. Place the normal shoot on M404 medium containing 1.5% sucrose, 2 g/L gelzan, and 150 mg/L timentin to encourage rooting.

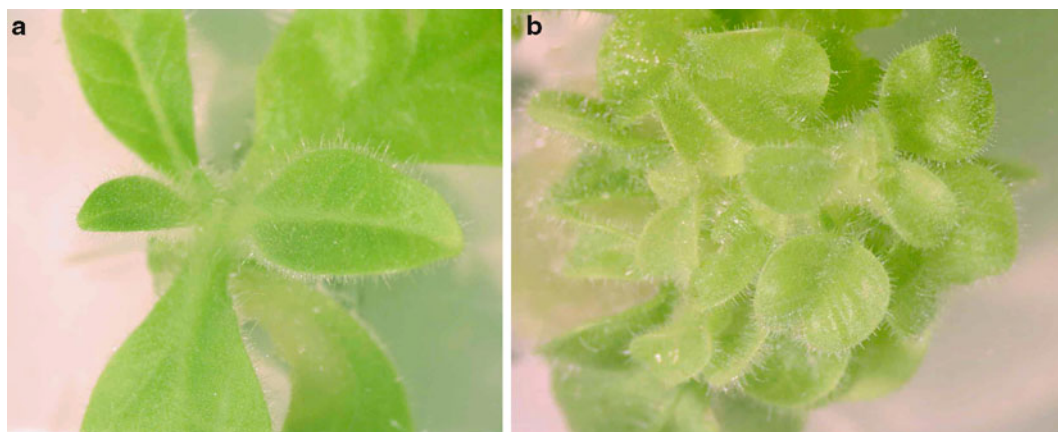


Fig. 1. Tobacco shoot phenotypes. (a) Normal shoot phenotype of a tobacco shoot arising from callus on regeneration medium. (b) Stunted, highly branched, and chlorotic tobacco shoot PCR-positive for the *ipt* gene. Photo was taken about 1 month after explant infection.

6. Once rooting occurs, collect tissue samples for DNA extraction.
7. Make PCR primer pairs to amplify the gene of interest.
8. Once gene-of-interest positive shoots are identified, carry out PCR screening to confirm the absence of vector backbone, preferably using primers to regions immediately downstream of the T-DNA left border. The frequency of marker-free, backbone-free shoots obtained using this method varies according to crop, the variety of crop, the complexity of the vector, and other unknowns. See Table 1 for expected transformation frequencies of the crop varieties tested.

4. Notes

1. *Agrobacterium* strain LBA4404 provided the most consistent and thorough infection of all the crop species subjected to our tests of this method while still being controlled easily by antibiotics utilized after co-cultivation. The C58C1 strain infects explants as well or better than LBA4404 but is more recalcitrant to antibiotic control subsequent to co-cultivation. Vectors need to be optimized so as to promote strong expression of the *ipt* gene, drive regeneration of plants, and cause the typical *ipt* overexpression phenotype typical of plants with stable integration of the *ipt* gene. For example, the ubiquitin-3 promoter (10) is characterized by strong constitutive expression in solanaceous and cruciferous crops. We also recommend placing the

ipt gene in the vector backbone as closely to the left border as possible. In the event of imprecise cutting at the LB, the *ipt* gene will identify the plants with even a small portion of the backbone integrated.

2. Although the majority of potato varieties regenerate well on hormone-free medium, the addition of small amounts of auxin or cytokinin may improve regeneration and transformation efficiencies. The amount of hormone is not sufficient to promote cells of untransformed explants to proliferate and regenerate spontaneously. We found that only one potato variety does not regenerate on hormone-free medium. In that case, small amounts of the auxin indole acetic acid (IAA) were needed to induce regeneration. For all other varieties tested, however, no addition of hormones is required to get *ipt*-driven regeneration of marker-free, backbone-free shoots.
3. Using the *ipt*-based method described here, the choice of explant material has an important effect on marker-free transformation frequencies. When we used tomato cotyledon explants as starting material, only callus formed on the explants over the duration of the trial. Shoots never arose from the calli growing from tomato cotyledon explants. Perhaps the addition of auxin for some time interval would remedy this problem, but we have not optimized this method because of the success using the hypocotyl starting material.
4. Choice of gelling agent in the regeneration medium can prove important. We discovered that replacing agar with gelzan accelerated the regeneration time needed for every potato variety tested. The shoots arising on gelzan tend to be more robust, and the *ipt*-positive phenotype is more distinguishable from the phenotype of shoots without the stable integration of *ipt*. This fact provides for a more accurate screening step. After 1 month on hormone-free medium, explants are transferred to fresh hormone-free medium.
5. To familiarize oneself with the phenotype associated with *ipt* overexpression in a particular crop species, it is advised to perform a transformation using a binary vector that has an *ipt* expression cassette and selectable marker gene within the borders of the transfer DNA. Use a regeneration medium and protocol proven to cause regeneration of shoots. The *ipt* abnormal phenotype can also be seen by putting young, unrooted shoots on a medium containing high levels of cytokinin for 2–4 weeks.

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Chapter 2

Organophosphorus Hydrolase: A Multifaceted Plant Genetic Marker Which Is Selectable, Scorable, and Quantifiable in Whole Seed

T. Scott Pinkerton, James R. Wild, and John A. Howard

Abstract

Organophosphorus hydrolase (OPH, EC 3.1.8.1) provides a novel function as an alternative genetic marker system for use in many types of plant transformations. OPH is a high-capacity hydrolase with multiple organophosphorus substrates, many of which are neurotoxins and thus used extensively as pesticides. This spectrum of organophosphates includes compounds that are phytotoxic as well as those that are hydrolyzed to products that are easily detected visually without significant disruption of plant health. This dichotomy gives OPH the features of both a selectable marker as well as that of a scorable marker system, and these characteristics have been tested at several stages during the plant transformation and regeneration process. Finally, it is possible to quantify hydrolytic activity in the seed without interfering with its subsequent growth and regeneration.

Key words: Plant transformation, Selectable marker, Scorable marker, Organophosphate pesticides and herbicides, Organophosphorus hydrolase, Coumaphos, Bensulide, Paraoxon, *Zea mays*

1. Introduction

The successful production of recombinant plants requires determination of whether the transgenic construct has been incorporated into the host genome. If the transformation frequency is near 100%, one can select any plant and demonstrate the presence of the transgene. Unfortunately, most whole plant transformation frequencies systems are inefficient, with a rate as low as 12% considered a good transformation frequency in maize (1). Therefore, the need for a method to detect the integrated foreign DNA without having to screen large numbers of plants persists.

1.1. Selectable Genetic Markers

The most practical method for transgene detection is the inclusion of a marker gene that provides positive or negative selection of transgenic material or a gene that provides a scorable visual cue. A wide range of genes that provide resistance to plant toxins have been used as positive selectable markers in plants (Table 1) (2). These include genes that provide resistance to broad-spectrum antibiotics or which have the ability to degrade and/or provide resistance to metabolic inhibitors (3–7). There are also a number of negative selectable markers that can be used to identify transgenic plants. For instance, the inclusion of a bacterial cytosine deaminase gene can result in negative selection of transgenic material if it is grown on media containing 5-fluorocytosine (8). Cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which is then converted into the thymidylate synthase inhibitor 5-fluorodUMP. The net result is that the cell is not able to produce dTTP for DNA synthesis which is required for growth. The disadvantage of negative selection agents is that they may cause serious damage to the health of the transgenic tissue. If the tissue is needed for further study and development, duplicate cultures or lines must be grown and tracked to ensure identification and recovery of transgenic material.

The use of selectable markers has become widespread in agricultural biotechnology as a means to incorporate agronomic and end-user traits. Antibiotic-resistance genes have dominated selectable markers for microbes and have been used successfully for plants (9). However, for transgenic plants, the most commonly used markers are those that confer herbicide resistance (10). Herbicide-resistance genes have a major advantage in that in addition to selecting cell lines in culture, the herbicides are designed to be used on whole plants. Resistance genes for many of the commonly used herbicides have been identified which also provides the opportunity to use the herbicides on transgenic commercial crops (2).

Table 1
Examples of chemicals and genes used for positive and negative selection in plants

Selection chemical	Chemical type	Marker gene	Marker selection	Reference
Neomycin	Antibiotic	<i>neo, nptII</i>	Positive	(3)
Kanamycin	Antibiotic	<i>aphA1</i>	Positive	(4)
Hygromycin	Antibiotic	<i>hph</i>	Positive	(5)
Phosphinothricin	Herbicide	<i>pat, bar</i>	Positive	(6)
Glyphosate	Herbicide	<i>EPSP synthase, gox</i>	Positive	(7)
5-Fluorocytosine	Toxic nucleotide precursor	<i>codA</i>	Negative	(8)

Some common examples include (1) glyphosate resistance, achieved through the use of resistant 5-enolpyruvylshikimate synthases (EPSP synthase) and/or the enzyme glyphosate oxidoreductase (gox) (7); (2) glufosinate resistance, achieved by the enzyme phosphinothricin *N*-acetyltransferase which acetylates glufosinate which prevents herbicidal action (6); and (3) chlorsulfuron resistance, achieved by using the enzyme acetolactosynthase (11).

1.2. Scorable Genetic Markers

Scorable markers or reporter genes provide a useful method for identifying gene expression. Separation of transgenic and non-transgenic materials can be achieved after the cells and plants have grown. Commonly used scorable marker genes include green fluorescent protein (GFP), firefly luciferase (ff-LUC), and bacterial β -glucuronidase (GUS). This is due to products that allow for detection by visual means (ff-LUC and GUS) or due to the properties of the protein itself (GFP) (12). They are particularly useful for the analysis of promoter and targeting systems being used in transgenic plant production. The drawback of these systems is that they do not provide for positive selection, and so many more cell lines and plants must be screened for the presence of the gene. This can be particularly problematic in tissue culture where transformed cells are not readily separated from non-transgenic cells.

1.3. Combined Scorable and Selectable Genetic Marker

We looked at an alternative marker system using the enzyme organophosphorus hydrolase (OPH, EC 3.1.8.1, aryldialkylphosphatase) that has the potential to provide both scorable and selectable phenotypes. OPH is a dimeric enzyme that is found in a number of bacterial species that have been shown to degrade synthetic organophosphates (13). The enzyme utilizes divalent transition metals as co-factors and has been shown to have a wide ranging activity against a number of organophosphate neurotoxins (see Note 1). The enzyme also shows a remarkable thermodynamic stability and has enzymatic activity over a wide range of conditions (14). While organophosphates are primarily thought of as neurotoxins, they also function as phytotoxins with a number of organophosphate compounds being used as commercial herbicides with different modes of action. These include the lipid synthesis inhibitor bensulide, the microtubular assembly inhibitors amiprofosmethyl and butamiphos, and the cell division inhibitors anilofos and piperophos (15–17). There are also structural similarities between known herbicides and compounds currently used for insect control, such as that of coumaphos and *O*-(2-naphthyl), *O'**O''*-diethyl phosphorothionate, a compound that has also been shown to have herbicidal activity (18, 19).

OPH has previously been used as a scorable marker in bacteria, utilizing the products of the OPH degradation of paraoxon and coumaphos as colored or fluorescent markers for the expression of the enzyme (19, 20). The wide range of potential OPH substrates,

including those with herbicidal activity, coupled with the previous use of OPH as a scorable marker system in bacteria make OPH an interesting subject of investigation as a marker gene for use in plant systems. To this end, the bacterial OPH gene was optimized for expression in a plant system, specifically *Zea mays*. This was achieved by the building of the optimized OPH gene from oligonucleotides with codon usage mimicking that found in maize. The protein was also fused to the barley alpha-amylase signal sequence which resulted in the export of the recombinant protein outside of the cell membrane. Other targeting sites may also work, but all marker experiments were carried out with this gene and signal sequence.

The use of OPH as a marker can be broken down into two distinct areas: experiments showing OPH utility (1) as a scorable marker and (2) as a selectable marker. The scorable marker experiments include both destructive and non-destructive assays for scoring transgenic callus using the insecticide coumaphos and related compounds, the scoring of intact seeds using the compound paraoxon, and the scoring of leaf tissue using coumaphos. The utility of OPH as a selectable marker in tissue culture was tested with several compounds, and the best results were obtained with the coumaphos derivative haloxon. Regenerated transgenic plants were also tested for resistance to the commercial organophosphate herbicide bensulide with resistance seen in transgenic plants at the recommended application rate for the commercial herbicide.

1.4. Future Prospects

OPH offers an exciting new selectable/scorable marker system for plants. It has the unique ability to act both a selectable marker using herbicidal organophosphorus compounds and as a scorable marker using the OPH substrates paraoxon and derivatives of coumaphos. The selectable marker activity of OPH has been shown against herbicides and herbicide-like compounds that have not been used commonly as selection agents. This may make OPH a useful marker for use in the production of transgenic plants where susceptibility to common broad-spectrum herbicides is still desired. For instance, OPH may find use in the production of plants producing industrial or pharmaceutical proteins. The use of OPH in this instance would allow for effective selection while retaining susceptibility to broad-spectrum herbicides such as glyphosate and glufosinate, which could be used as control agents during field trials to prevent environmental escape of the unwanted transgenic plants. The scorability of the OPH system also gives a rapid way to detect transgenic plants, which could also be useful during the production of transgenic plants. OPH also has a great deal of potential that may be realized by engineering the enzyme itself. Alterations of the OPH active site may yield new or better activity that can be used in the selection and scoring process. Given the relatively rare use of organophosphate herbicides in comparison to herbicides like glyphosate, the OPH marker gene may be especially useful when resistance to a broad-spectrum herbicide is not desired.

2. Materials

Unless stated otherwise, all reagents and chemicals were of the highest purity available. Water used was deionized and filtered. The *opd* gene sequence used was derived from the bacterium *Brevundimonas diminuta* as described by Dumas et al. (21). The *opd* gene sequence was optimized as described in Pinkerton et al. (22).

2.1. Supplies and Equipment

Standard equipment used included a laminar flow hood, spectrophotometer/plate reader, UV illuminator or transilluminator with long wavelength UV capability, 24- or 48-well tissue culture plates, 27°C incubator, and pipettes.

2.2. Reagents, Stock Solutions, and Media

1. 100 mg/mL Coumaphos in dimethyl sulfoxide (DMSO).
2. 0.1 mM Coroxon in 50 mM Tris-HCL buffer (pH = 7.4).
3. 1 mM Paraoxon in 10 mM Tris-HCL buffer (pH = 7.2).
4. 100 mg/mL Haloxon in dimethyl sulfoxide (DMSO).
5. Commercial bensulide-based pesticides Bensumec-4LF or Prefar at manufacturer's recommended application concentration by dilution in acetone.
6. Tissue culture media (specific to type of culture).

All solutions were filtered through 0.22-μm filters prior to use.

3. Methods

3.1. Scoring of Transgenic Callus Using Coumaphos and Related Compounds

Coumaphos together with its related oxon derivative coroxon and the chlorinated derivative haloxon are all OPH substrates (see Notes 2 and 3). Cleavage of these compounds by OPH results in the release of 3-chloro-7-hydroxy-4-methyl-chromen-2-one which is fluorescent under UV illumination. It is this fluorescence that is readily visible in tissue culture and indicates the presence of a transgene. Our results showed that coumaphos generally gave a weaker signal compared to those of coroxon and haloxon. This may be due to the stronger catalytic performance of OPH against the oxon derivatives of compounds compared to the thion form of a compound. The results of a callus screen with coumaphos, coroxon, and haloxon are shown in Fig. 1. Our results with 22 OPH-expressing callus lines indicate that lines expressing at a level of 0.01 units of activity/mg of soluble protein were detectable by this method:

1. Prepare a 100 mg/mL solution of coumaphos or one of its derivatives in DMSO.

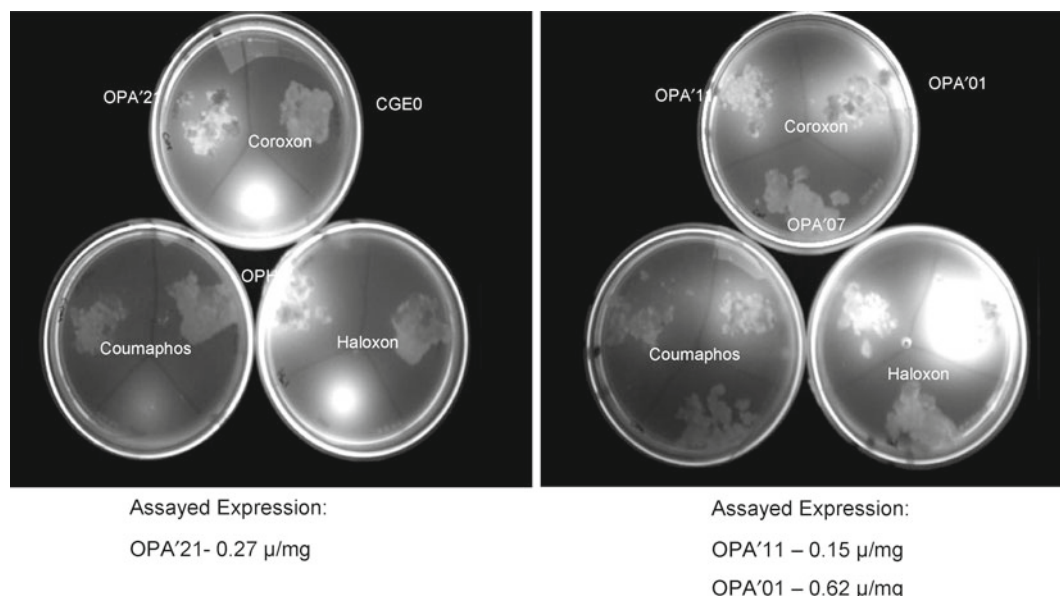


Fig. 1. Detection of transgenic callus with herbicide derivatives. OPA-transformed callus was plated onto tissue culture plates containing 1 mg/plate coumaphos, coroxon, or haloxon. After 24 h, the plates were illuminated with UV light. Fresh maize callus CGE07 provides a native, transgenic negative control, and recombinant OPH from *Escherichia coli* serves as a positive control.

2. Filter sterilize the solution through a 0.22-µm syringe filter.

When pouring the tissue culture media of choice, add the coumaphos solution to the plate prior to adding the media so that the final concentration of coumaphos in the media is 28.5 µg/mL.

3. After plates have been allowed to solidify, store at 4°C in the dark.
4. Plate the callus material to be tested onto the coumaphos plates.
5. Place the plates in an opaque plastic container and store at 27°C for 24 h.
6. After the storage period, place the plate on a short-wavelength UV transilluminator and observe any fluorescence.
7. Callus expressing the OPH protein will show a UV-induced fluorescence both in the media surrounding the tissue as well as within the tissue itself.

3.2. Scoring of Transgenic Leaf Tissue Using Coumaphos and Related Compounds

We found that the use of coumaphos and related compounds could be extended for use with excised leaf tissue. Again, the oxon derivatives tend to give a better signal. Results of a screening of transgenic leaf tissue are shown in Fig. 2:

1. Prepare a 0.1 mM solution of coroxon (the oxon derivative of coumaphos) in 50 mM Tris-HCL buffer (pH=7.4).

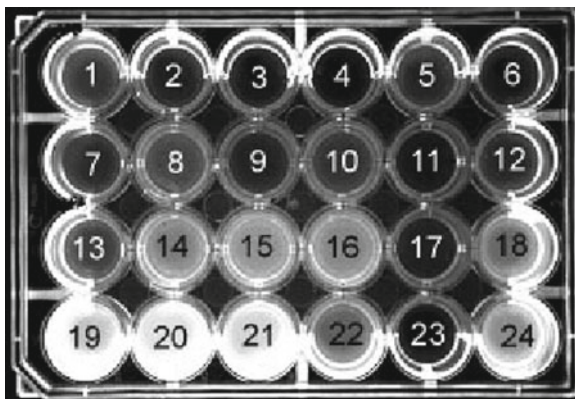


Fig. 2. Detection of OPH activity in leaf tissue with coroxon. Leaf tissue was incubated for 20 h in 1 mL of 0.1 mM coroxon. Wells 1–12 contained OPH-expressing maize tissue under the seed specific promoter; wells 13–24 contained OPH-expressing maize tissue under the control of a constitutive promoter. Well 11 had no leaf tissue and served as a negative control. Constitutive lines 19, 20, and 21 showed strong OPH activity by UV fluorescence.

2. Place 1 mL of the coroxon solution into each well of a 24-well tissue culture plate.
3. Excise a 25-mm square segment of leaf tissue from plants 3 weeks postgermination. This method works best when the OPH is being expressed under a constitutive promoter.
4. Float each segment in its individual well for 20 h at room temperature.
5. After the incubation period, remove the tissue and place the plate on a UV transilluminator.
6. OPH positive plants will show fluorescence under UV illumination.

3.3. Scoring of Transgenic Seed Using Paraoxon

The activity of OPH can also be used to screen seeds for presence of the marker prior to planting (see Note 4). This method is non-destructive, and seeds remain viable. The seeds are screened for the enzymatic activity of OPH towards paraoxon, the oxon derivative of the pesticide parathion. Enzymatic activity by OPH results in the cleavage of paraoxon and the release of *p*-nitrophenol, which has a strong yellow color at neutral-basic pHs. Results of a seed screening with paraoxon are shown in Fig. 3. Seed screening showed that those seeds that generated high *p*-nitrophenol absorbance had a high correlation (>95%) with resistance to bialaphos that was carried by secondary selectable marker (*pat*) included in the construct:

1. Prepare a solution of 1 mM paraoxon in 10 mM Tris-HCL buffer (pH=7.2).

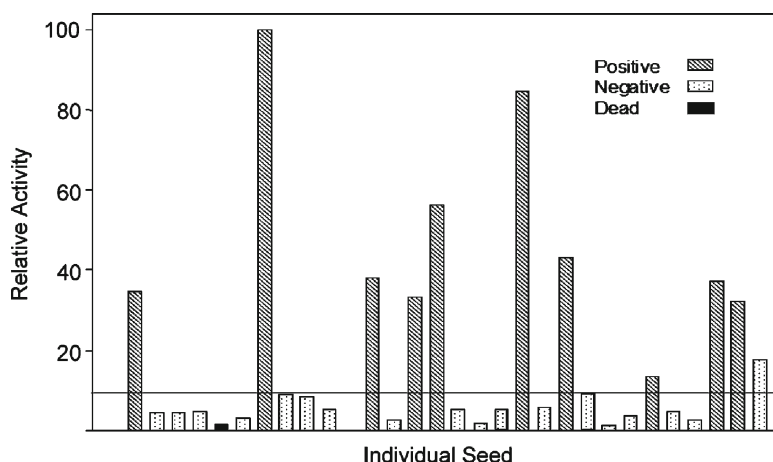


Fig. 3. Relative activity of OPH is plotted from 30 individual seeds. Seeds were evaluated (tested for *pat+*) by bialophos leaf painting of plants resulting from seed generation. Seed 5 did not germinate (dead). The background enzymatic activities (paraoxon hydrolysis of less than ± 0.05 ABS units at 400 nm) are indicated by the horizontal line at 8% relative activity. Each seed could be assayed once; the technical assay error is estimated to be $\pm 10\%$.

2. Place 1 mL of the paraoxon solution into each well of a 24-well tissue culture plate.
3. Place individual seeds into each well and incubate with shaking at room temperature for 3 h.
4. After 3 h, remove each seed, at which point the seeds should be planted.
5. A turning of the solution from clear to yellow is indicative of the presence of the transgene in the seed.
6. This yellow color can be quantified by measuring the absorbance of the reaction solution on a spectrophotometer at a wavelength of 400 nm.

Our experiments with this method included measurement of the intact seed activity compared to the extractable enzymatic activity from the seed. There was a general trend that the higher the activity in intact seeds the higher the amount of extractable activity from each seed.

3.4. Callus Selection Using Coumaphos Derivatives

During our experimentation with coumaphos derivatives for scoring transgenic callus, we noticed that necrosis was induced in control callus and callus that was low for expression of OPH. Coumaphos and its derivatives are structurally similar to compounds that have been patented for use as herbicides. Selection experiments were conducted with several other OP herbicides, but the best results were obtained with the coumaphos derivative

haloxon. The greatest differential in growth between transgenic and non-transgenic tissue on haloxon plates was seen at a concentration of 5 μM . Growth of callus on media containing two organophosphate herbicides and on media containing haloxon is shown in Fig. 4:

1. Make up a stock of haloxon at 100 mg/mL in DMSO.
2. Add the stock haloxon directly to the plate during the pouring of the tissue culture media at a concentration of 5 μM .

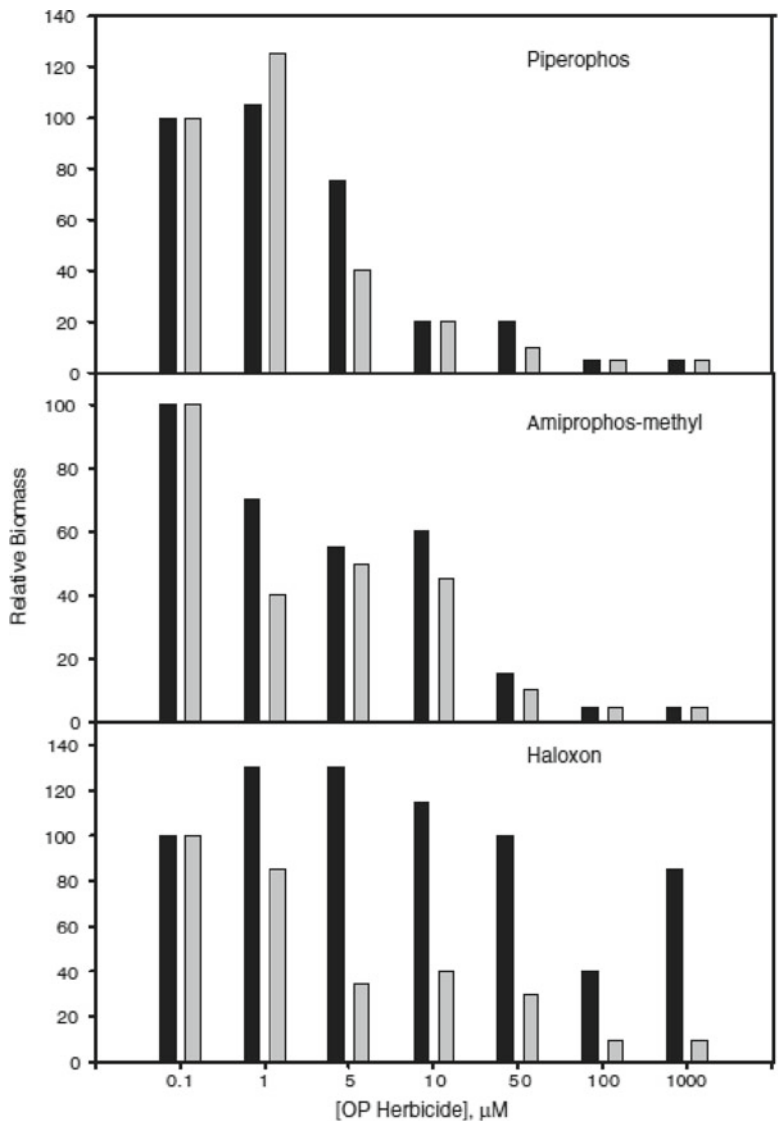


Fig. 4. The effect of OP herbicides on growth of transgenic OPH-maize callus. The *top panel* compares the effects of piperophos on growth (weighed biomass production); the *middle panel* summarizes effects of amiprofosmethyl; and the *lower panel* compares the effects of haloxon. Relative biomass is reported as the weight of native callus (*open bars*) and OPH-expressing callus (*shadowed bars*) over a 6-week growth period.

3. Transfer tissue for selection to haloxon plates.
4. Observe plate for new growth and necrosis.
5. After 2 weeks, transfer to fresh haloxon plates.

3.5. Selection of Plants Using Bensulide-Based Herbicides

During our search for potential herbicides for use in selection of regenerated plants, bensulide-based herbicides Bensumec-4LF and Prefar were the most readily available, and our work showed that the OPH-expressing plants were resistant to both commercial preparations at the recommended application rate (see Note 5). Both herbicides are pre-emergents, and they were added to the soil prior to planting. Results of transgenic plants growing on bensulide-treated soil are shown in Fig. 5. At the manufacturer's suggested application rate, the transgenic plants exhibited little to no apparent growth inhibition while a majority of the non-OPH-expressing control plants failed to germinate. The control plants that did germinate failed to develop a root system:

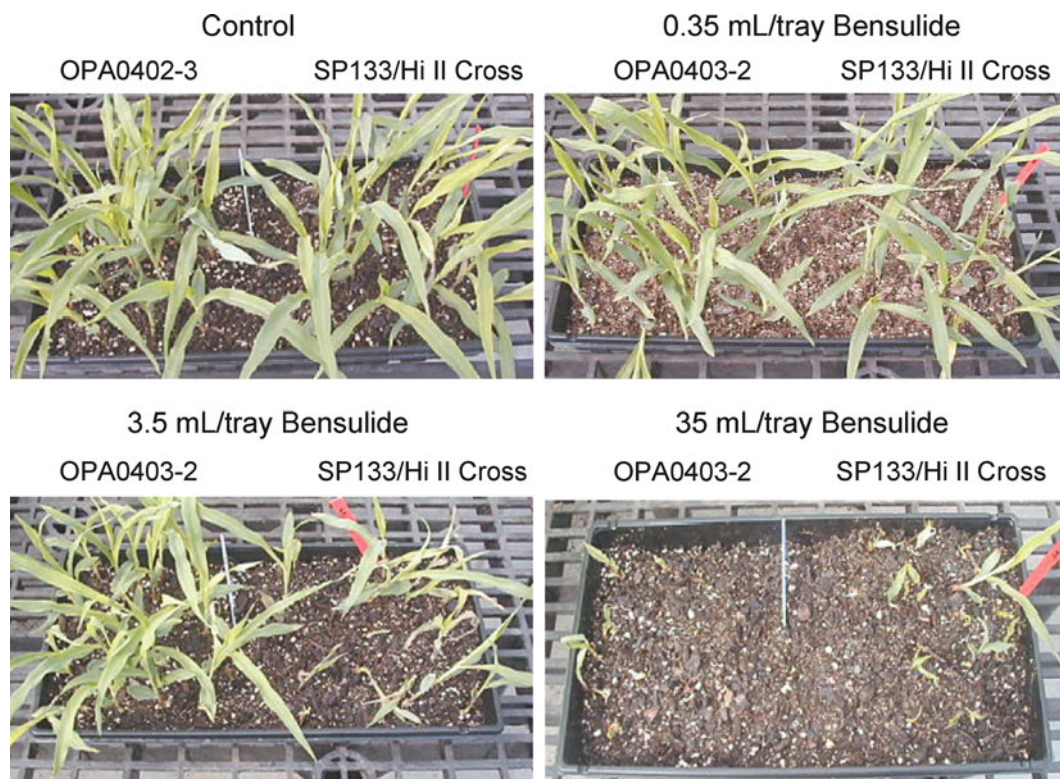


Fig. 5. Resistance of OPA plants to Bensumec-4LF (a bensulide formulation). OPA-0403 (OPH-expressing) seed was used to determine resistance to bensulide in the form of the formulated herbicide Bensumec-4LF. Control seed is of the same genetic background as the transgenic seed. Pictures were taken 3 weeks after germination. Shoots of control plants at 3.5-mL/tray treatment level as well as all shoots at the 35-mL/tray treatment level lacked a developed root system.

1. Determine the amount of the commercial herbicide needed to treat the soil area planted using the manufacturer's suggested application rates. For a standard 21"×11" germination tray, this is 3.5 mL of Bensumec-4LF.
2. Dilute the herbicide into acetone. For a germination tray, 300 mL of acetone was used.
3. Mix the diluted herbicide into the soil. We found that the easiest way to do this was to mix the herbicide solution into the pre-measured soil in a plastic autoclave bag. The herbicide was then distributed by mixing the soil well by kneading the outside of the bag.
4. Distribute the treated soil into its container and allow the acetone to evaporate for a period of at least 3 h.
5. Plant the seed for selection.
6. After germination, observe the plants for 3 weeks. Bensulide affects root growth, so it is possible for shoot development to occur without significant root growth in non-transgenic plants.
7. At 3 weeks, transplant to non-bensulide-containing soil. Plants with well-developed root systems should be transgenic.

4. Notes

1. While our early work with OPH as a marker in a plant system has shown remarkable promise, there is still much work to be done to refine and improve the system. Because OPH is a metalloenzyme, the effect of divalent transition metal ions in the cell culture systems would be of interest. Earlier results have shown that activity can be increased by 20-fold (22). It is possible that inclusion of cobalt or zinc within the selection media may result in increased enzyme performance and higher resistance to selection compounds. This may also affect the scoring systems with higher enzymatic output to differentiate transgenic from non-transgenic tissue.
2. One area of great potential is analysis of other OPH substrates for their potential use in a selection system. OPH has remarkable versatility and acts on several different types of bonds within organophosphate compounds, including P–O, P–S, P–F, and P–CN bonds. This gives OPH a wide range of substrates that may have the potential for use in plant systems. There are also a number of organophosphorus compounds that OPH has never been tested against. This includes a number of compounds that have been patented for their herbicidal activity (23–26).

3. Another potential area of study involves modification of the OPH enzyme itself. Previous studies with OPH have shown that directed engineering of the enzyme's active site can positively change the rate at which OPH will cleave P-S substrates. Alteration of active site residues within the enzyme could result in higher or new rates of activity against herbicidal substrates (27).

The areas discussed above could influence the function of OPH as a scorable marker. Given the wide range of potential compounds a number of different scoring agents may be possible. It is also possible that novel OPH substrates could be synthesized with products that allow for the use of other detection methods.

4. There is also the potential of analyzing other enzymes with organophosphate hydrolase activity for their utility as a selectable/scorable marker system. The *B. diminuta* enzyme is only one of several enzymes that have been identified with activity against organophosphates. These other enzymes include squid DFPase, the PON serum paraxonases, and a number of other bacterial enzymes (1). These enzymes may prove to have a utility similar to OPH.
5. The recent successful expression of OPH in tomato also expands the utility of OPH as a potential marker system in plants beyond *Zea mays* (28).

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Use of Northern Blotting for Specific Detection of Small RNA Molecules in Transgenic Plants

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Abstract

Small RNAs (20–24 nucleotides long and nonprotein coding) have been increasingly investigated. They are responsible for phenomena described as RNA interference (RNAi), cosuppression, gene silencing, or quelling. Major classes of small RNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs), which differ in their biosynthesis. MiRNAs control the expression of cognate target genes by binding to reverse complementary sequences, resulting in cleavage or translational inhibition of the target RNA. SiRNAs have similar structure, function, and biogenesis as miRNAs; siRNAs derive from long double-stranded RNA of transgenes, endogenous repeat sequences, or transposons. Understanding these fundamental processes requires the sensitive and specific detection of small RNA species. In this report, we present a simple Northern blot protocol for small RNAs in transgenic plants.

Key words: Small RNA, miRNA, siRNA, Northern, Blotting, Transgenic plants

1. Introduction

RNAi is a mechanism regulating gene transcript levels by either transcriptional gene silencing (TGS) or by post-transcriptional gene silencing (PTGS), which acts in genome maintenance and the regulation of development (1). Since the discovery of RNAi in *Caenorhabditis elegans* (2, 3), extensive studies have been performed focusing on the different aspects of RNAi. In particular, the elucidation of the essential components of RNAi pathways has advanced extensively (1, 4). RNAi has been discovered in a wide range of organisms from plants and fungi to insects and mammals, suggesting that it arose early in the evolution of multicellular organisms (5).

Small noncoding RNAs (20–24 nucleotides in size) have been increasingly investigated, and they are important regulators of PTGS in eukaryotes (6, 7). They were first discovered in the

nematode *C. elegans* (3), and they are responsible for phenomena described as RNAi, cosuppression, gene silencing, or quelling (8–10). Shortly after these reports were published, it was shown that PTGS in plants is correlated to small RNAs (7). These small RNAs regulate various biological processes, often by interfering with mRNA translation. Based on their biogenesis and function, small RNAs are classified as repeat-associated small interfering RNAs (ra-siRNAs), trans-acting siRNAs (ta-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), and microRNAs (miRNAs) (5). Major classes of small RNAs include miRNAs and siRNAs, which differ in their biosynthesis (5, 11). MiRNAs are ~21-nt-long small RNAs transcribed from endogenous MIR genes which form precursor RNAs with a characteristic hairpin structure, generated from processing of longer pre-miRNA precursors by DICER-LIKE or DCL in plants. These miRNAs are recruited to the RNA-induced silencing complex (RISC) (11, 12). Recently, miRNAs have been identified as important regulators of gene expression in both plants and animals (13), and they are highly conserved in evolution (13–16). SiRNAs have similar structure, function, and biogenesis as miRNAs; siRNAs derive from long double-stranded RNA of transgenes, endogenous repeat sequences, or transposons (17, 18). *Trans-acting* small interfering RNAs (ta-siRNA), like miRNAs, are originated from loci that give rise to noncoding transcripts that themselves are targets of miRNAs and guide the cleavage of specific mRNA (5, 19). These small RNAs are now being developed into tools for biological research.

The detection of small RNAs is an important step in the understanding of their role and function. Northern analysis is a widely used method for small RNA detection and analyses because it is generally a readily available technology for laboratories and does not require special equipment and technical knowledge. Here, we report the optimization of small RNA Northern blot detection method for transgenic plants.

2. Materials

2.1. Isolation of Total RNA from Plants Using TRIzol® Reagent

1. Liquid nitrogen.
2. TRIzol® reagent (Invitrogen, Karlsruhe).
3. Chloroform.
4. Isopropanol.
5. 75% Ethanol in DEPC-treated H₂O.
6. RNase-free water (GIBCO).

2.2. Small RNA Northern Blotting (Detection and Analysis of Small RNAs)

1. Electrophoresis system.
2. Polyacrylamide gel: 40% acrylamide solution; TBE buffer (0.9 M Tris-HCl, 0.9 M boric acid, 0.02 M EDTA, pH 8.0); 8.3 M urea; 10% APS (immediately freeze in aliquots for single use at -20°C); N,N,N,N' -tetramethylethylenediamine (TEMED, pH 7.0).
3. Electrophoresis buffer: $0.5\times$ TBE.
4. Small RNA loading dye ($2\times$): 10 mL deionized formamide, 200 μL 0.5 M EDTA (pH 8.0), 10 mg xylene cyanole, 10 mg/mL bromophenol blue.
5. Labeled decade marker RNA (100 ng/ μL ; Ambion), stored at -20°C .
6. Hybond N^+ nylon membrane (GE Healthcare).
7. Whatman paper: GB002 grade.
8. Electroblotter: Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad).
9. UV cross-linker (Biolink BLX, Biometra).
10. DNA oligonucleotides complementary to small RNA molecules.
11. [$\gamma^{32}\text{P}$]-ATP: ~ 6.000 Ci/mmol; 10 $\mu\text{Ci}/\mu\text{L}$.
12. T4 polynucleotide kinase (10 U/ μL ; Fermentas).
13. Nucleotide Removal Kit (QIAGEN).
14. Hybridization buffer: 0.05 M sodium phosphate (pH 7.2), 1 mM EDTA, $6\times$ SSC, $1\times$ Denhardt's, 5% SDS.
15. Washing solutions: $2\times$ SSC, 0.2% SDS and $1\times$ SSC, 0.1% SDS.
16. Phosphoimager (Imager FX, Bio-Rad).

3. Methods

3.1. RNA Extraction

The extraction of intact, high-quality RNA is a prerequisite for the reliable detection of small RNAs. TRIzol[®] reagent (Invitrogen) is well suited for purifying RNA samples of appropriate quality for small RNA detection (see Notes 1 and 2).

1. This protocol is designed for extraction of large amounts of RNA.
2. Consider that some tissues contain more RNAs than others. Inflorescences and seedlings are very rich in RNA, while older leaves contain much less.
3. Grind 0.1 g plant tissue in liquid nitrogen (mortar and pestle); use less tissue for small-scale protocol (too much tissue negatively affects the purity of the RNA).

4. Immediately add TRIzol reagent (1 mL TRIzol to 50–100 mg frozen tissue) before tissue thaws and thoroughly mix with powder tissue. The mixture might freeze (prealiquoting TRIzol in 15-mL Falcon tubes allows you to proceed much faster at this step).
5. Incubate for 5 min at room temperature until the TRIzol–tissue mixture is completely liquid again (dissociation of nucleoprotein complexes).
6. Centrifuge for 10 min at $5,000\times g$ at 4°C . This step pellets excess particles; proceed only with the liquid phase so that chloroform extractions become much easier and less numerous.
7. Decant supernatant into new Falcon tube.
8. Add 0.2 mL chloroform per initial milliliter of TRIzol and mix well.
9. Incubate for 5 min at room temperature.
10. Centrifuge for 30 min at $5,000\times g$ at 4°C until clear phase separation.
11. Transfer upper phase into new Falcon tube.
12. Add an equal volume isopropanol and mix.
13. Precipitate at -20°C overnight.
14. Centrifuge for 30 min at $5,000\times g$, 4°C (yields white RNA pellet).
15. Discard the supernatant and wash the pellet with 1 mL 75% ethanol per initial millilitre TRIzol.
16. Centrifuge for 8 min at $5,000\times g$, 4°C .
17. Remove the supernatant quantitatively and briefly air-dry pellet (no longer than 10 min).
18. Resuspend the pellet in about 50 μL of DEPC-treated water. (Volume depends on the amount of RNA you expect. Higher concentrations are favorable as this allows the use of a smaller or lower volume on gel).
19. To dissolve RNA in water, keep on ice for a while and heat to 65°C in a water bath once or twice.
20. Quantify RNA with a photometer.
21. It may be necessary to run a regular gel to make sure that the RNA is not degraded.

3.2. Small RNA Northern Blotting (Detection and Analysis of Small RNAs)

Before starting, wash all equipment with NaOH (incubate for about 2–4 h) and wash thoroughly with water (see Note 3). Do not leave in NaOH for too long (days) because glues might dissolve. To prove the accumulation of the small RNAs in the plant lines, you should perform a small RNA gel blot analysis with an antisense probe for the small RNAs.

1. Preparation of polyacrylamide gel mix: 12 mL acrylamide solution (40%), 4 mL TBE buffer (10×), 20 g urea (8.3 M), 320 μ L APS (10%), and 42.4 μ L TEMED; adjust the volume to 40 mL with H₂O (see Note 3).
2. Allow gel to polymerize for 1 h.
3. Assemble gel apparatus (see Note 4) and add the running buffer (0.5× TBE).
4. Clean wells with running buffer and make sure there are no leaks.
5. Load 50–70 μ g of total RNA per lane and adjust the volume to 10 μ L with DEPC-H₂O.
6. Add 10 μ L of 2× RPA loading buffer to your RNA sample.
7. Heat RNA at 65°C for 10 min.
8. Chill on ice for 1 min.
9. Load samples and run at 60 V for 16 h or until the bromophenol blue reaches the bottom of the gel.
10. After electrophoresis, stain the gel with ethidium bromide in 0.5× TBE for 30 min. This will allow you to visualize tRNAs and 5S RNA to check for RNA integrity.
11. Rinse gel in 0.5× TBE to remove excess of ethidium bromide.
12. Cut the Hybond N⁺ nylon membrane to a size slightly larger than the gel.
13. Soak the membrane in transfer buffer (0.5× TBE) for 1 min.
14. Soak four pieces of Whatman paper (GB002 grade) in 0.5× TBE.
15. Set up transfer in the Trans-Blot Semi-Dry Electrophoretic Transfer Cell as such from bottom (anode) to top (cathode): two Whatman papers (GB002 grade), Hybond N⁺ nylon membrane, gel, and cathode plate. Make sure to roll out any bubbles.
16. Transfer the RNA at 400 mA for 1 h. The voltage will start out low but will increase by the end of the transfer.
17. Wash blot in 0.5× TBE to remove any traces of the gel.
18. Place wet membrane on a wet sheet of filter paper and UV cross-link at optimal setting.
19. Store membrane dry until use.
20. For prehybridization, use 50 mL of hybridization buffer. Prehybridization should be carried out for at least 2 h.
21. The prehybridization and hybridization temperatures depend on the sequence of the small RNA antisense oligonucleotides. Calculate the T_m (annealing temperature) of the oligonucleotide using the following formula (this formula is

valid for small oligonucleotides with a size of 17–24 nt): $T_m = (A + T) \times 2 + (C + G) \times 4$. The hybridization temperature should be 10°C below the calculated T_m of the oligonucleotide.

22. Oligonucleotide labeling reaction:

- 1 μ L oligonucleotide (10 pmol/ μ L).
- 11 μ L H₂O.
- Heat for 5 min at 75°C and cool 3 min on ice.
- Add 2 μ L reaction buffer A (supplied Fermentas).
- Add 5 μ L [γ^{32} P]ATP (10 μ Ci/ μ L).
- Add 1 μ L T4 polynucleotide kinase (10 U/ μ L; Fermentas).
- Mix well with pipette and incubate 60 min at 37°C.
- Stop the labeling reaction by heat inactivation of the enzyme for 10 min at 68°C.

23. Purify the labeled oligonucleotide using the Nucleotide Removal Kit (QIAGEN) and elute the oligonucleotide from the column by eluting twice with 150 μ L EB buffer and combine eluates.

24. Before adding the labeled oligonucleotide to the hybridization buffer, heat for 5 min at 75°C. Before hybridization, exchange the prehybridization buffer and hybridize in 20 mL (see Note 5) of fresh hybridization buffer for 12–18 h.

25. Wash the membrane: Washing is crucial and there are no general rules (washing steps depend strongly on T_m of the hybridization probe, expression level of the small RNA, etc.). Washes should be performed at a temperature 10°C below the calculated T_m of the oligonucleotide probe:

- Discard hybridization solution.
- Wash briefly with 2 $\times$ SSC/0.2% SDS.
- Wash twice for 10 min with 2 $\times$ SSC/0.2% SDS (check signals on the membrane with a Geiger tube; if necessary, continue washing).
- Wash for 5–10 min with 1 $\times$ SSC/0.2% SDS (check signals on the membrane with a Geiger tube; if necessary, continue washing and increase washing temperature moderately).

26. Wrap membrane with Saran wrap and expose to a phosphorimager to detect hybridization signals.

27. If membranes need be used for additional hybridizations (e.g., control hybridization for normalization), the membranes have to be stripped to remove bound radioactively labeled probes. Prepare 0.1% SDS and heat the solution to 95°C. Wash the

membrane two times for 15 min at 90°C. Expose the membrane for at least 24 h to control complete removal of the probe. Stripped membranes can be frozen at -20°C wrapped with Saran wrap and can be reused several times.

28. Use an antisense U6snRNA probe (5'-GGGGCCATGCTAA TCTTCTCTG-3') for control hybridization. This probe was end-labeled using the PNK method and hybridized to the membrane as described above.
29. We highly recommend the use of ³²P-labeled RNA markers such as the decade ladder from Ambion (#7778). Not only do they provide a good size range, but they also function as internal indicators of how well RNA was transferred in the blot and (later) how much RNA was cross-linked and retained on the membrane after hybridization and wash. For example, during blotting, you can monitor the process by peeling back the membrane slightly from the gel (at the corner where the largest RNA markers should be) and comparing the counts still in the gel with those now on the membrane.

4. Notes

1. Be careful working with RNA; RNA is easily degraded by RNAases which seem to be present everywhere. Therefore, we recommend working in a clean lab environment when you handle RNA.
2. You cannot use regular RNA columns (e.g., RNeasy columns from QIAGEN) to extract small RNAs since their size exclusion limit is too large (small RNAs are lost). Use either TRIzol reagent (Invitrogen) or specialized columns (e.g., the mirVana kit from Ambion).
3. Use autoclaved pipette tips and baked glassware. Wear gloves when you work with RNA. In most lab manuals, it is recommended to prepare all required solutions with DEPC-treated H₂O.
4. To guarantee a good separation of the RNA samples, we recommend using a Midi-Gel rather than using Mini-Gels. Furthermore, if high amounts of RNA (>10 µg of total RNA) are loaded onto the gel, it is better to use a 10- or 12-well comb. When using a 20-well comb, the amount of RNA per well might be too much and may result in bad signals after the hybridization.
5. Hybridization conditions may differ substantially based on the intention of the designed experiment. The most important

criteria are the stringency conditions. High hybridization and wash temperature coupled with low salt concentrations are high-stringency hybridization conditions. Low temperature and high salt concentrations favor unspecific hybridizations and represent low-stringency hybridization conditions. Keep the volume of hybridization buffer as small as possible to achieve high probe concentration during the hybridization; the membrane has to be covered completely.

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Part II

Transformation Technology: Lower Plants

Chapter 4

Genetic Transformation of the Model Green Alga *Chlamydomonas reinhardtii*

Juliane Neupert, Ning Shao, Yinghong Lu, and Ralph Bock

Abstract

Over the past three decades, the single-celled green alga *Chlamydomonas reinhardtii* has become an invaluable model organism in plant biology and an attractive production host in biotechnology. The genetic transformation of *Chlamydomonas* is relatively simple and efficient, but achieving high expression levels of foreign genes has remained challenging. Here, we provide working protocols for algal cultivation and transformation as well as for selection and analysis of transgenic algal clones. We focus on two commonly used transformation methods for *Chlamydomonas*: glass bead-assisted transformation and particle gun-mediated (biolistic) transformation. In addition, we describe available tools for promoting efficient transgene expression and highlight important considerations for designing transformation vectors.

Key words: Green alga, *Chlamydomonas reinhardtii*, Transformation, Biolistic transformation, Particle gun, Reporter gene, Selectable marker gene, Expression strain, Autolysin, Luciferase activity assay

1. Introduction

Chlamydomonas reinhardtii is a unicellular motile green alga that swims with two flagella (1, 2). It belongs to the class Chlorophyceae within the order Volvocales. The *Chlamydomonas* cell is surrounded by a wall of hydroxyproline-rich glycoproteins and contains a single large cup-shaped chloroplast that occupies approximately two-thirds of the cell volume. Under favorable environmental conditions, the (haploid) alga usually reproduces asexually by simple mitotic cell divisions that are initiated once a critical cell size has been reached. Starvation (especially nitrogen limitation) triggers the onset of sexual reproduction in which the algal cells can undergo differentiation into gametes followed by syngamy between two gametes of opposite mating types (mt+ and mt-).

The diploid zygote germinates by meiosis, releasing four haploid cells that, under favorable conditions, continue to reproduce asexually by mitosis.

Chlamydomonas has many attractions that favored its adoption as a model system in plant biology. The alga can be easily grown at large scale either photoautotrophically, mixotrophically, or heterotrophically (using acetate as organic carbon source). In many ways, *Chlamydomonas* genetics is similarly simple to and as powerful as yeast genetics. Over the past decades, large mutant collections have been established, and all three genomes (nuclear, plastid, and mitochondrial) are amenable to genetic manipulation by transformation (3–6), which gives *Chlamydomonas* a unique status among plants. Thanks to a great community effort, all three genomes are now fully sequenced (7). This, as well as the availability of efficient methods for chemical mutagenesis, insertional mutagenesis (8), RNA interference (RNAi; see ref. 9), and gene silencing by artificial microRNAs (amiRNAs; see refs. 10, 11), has made the alga a superb model system in plant post-genomics research.

Over the past decades, research on *Chlamydomonas* has made seminal contributions to a number of fields, including chloroplast biology, photosynthesis research, photobiology (light sensing and signal transduction), and the structure and function of flagella (1, 3, 12, 13). More recently, the alga has also attracted great interest from biotechnologists, both as a potentially inexpensive production system for recombinant proteins (an area commonly referred to as molecular farming) and a promising production host for next-generation biofuels, such as hydrogen or biodiesel (14, 15).

One of the few limitations in *Chlamydomonas* research has been the notorious difficulty with expressing foreign genes from the nuclear genome to reasonably high levels. The genetic transformation process itself appears to be highly efficient (6); however, the transgene expression levels are often disappointingly low (16, 17). For many years, this has severely hampered both post-genomics research with *Chlamydomonas* and the exploitation of the alga as a production platform in biotechnology. Recently, the use of specialized promoters and reporter genes, as well as the isolation of mutant algal strains that express foreign genes to higher levels, has alleviated this problem, but the molecular and genetic basis of the low efficiency of transgene expression from the *Chlamydomonas* nuclear genome remains elusive.

Here, we provide protocols for the efficient transformation of *C. reinhardtii* and describe strategies for successful transgene expression from the alga's nuclear genome.

2. Materials

2.1. Algal Culture

Unless otherwise stated, *C. reinhardtii* cells are grown photomixotrophically either in liquid or on solid TAP medium (18) at 22–24°C under a 16-/8-h day/night cycle with 50 $\mu\text{mol}/\text{m}^2/\text{s}$ light intensity. Aeration of liquid cultures should be secured by continuous shaking at 120 rpm. If the arginine auxotrophic strain cw15-302 is used, arginine has to be added to the medium at a final concentration of 100 $\mu\text{g}/\text{ml}$ shortly before inoculation.

2.2. Chemicals and Stock Solutions for Culture Media

1. Phosphate buffer (for 1 L: 108 g K_2HPO_4 and 56 g KH_2PO_4 ; add water to final volume of 1 L).
2. Nutrient stock solution I (for 1 L: 40 g NH_4Cl , 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; add water to final volume of 1 L).
3. Nutrient stock solution II (for 1 L: 55.7 g KCl , 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; add water to final volume of 1 L).
4. Hutner's trace metal solution (for 1 L: 11.4 g BO_3H_3 , 22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5.06 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4.99 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.61 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.57 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.1 g $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6 \cdot 4\text{H}_2\text{O}$, and 50 g EDTA; see Note 1).
5. Glacial acetic acid.
6. Plant cell culture-tested agar (Sigma-Aldrich).

2.3. Algal Culture Media

1. TAP (Tris-acetate phosphate) medium. Add 20 ml 1 M Tris, 1 ml phosphate buffer, 1 ml Hutner's trace metal solution, and 10 ml nutrient stock solution I to approximately 0.5 L of bi-distilled water, adjust pH to 7.0 with glacial acetic acid, and add water to a final volume of 1 L. Prepare TAP solid medium by adding 1.5% plant cell culture-tested agar prior to autoclaving of the medium.
2. TAP-N (nitrogen-deficient medium for induction of gametogenesis). For 1 L TAP-N, prepare TAP as described above but replace nutrient stock solution I with nutrient stock solution II.

2.4. Materials for Glass Bead Transformation

1. Glass beads (acid washed, 425–600 μm , Sigma-Aldrich).
2. Standard laboratory vortex.
3. Linearized transformation vector.
4. TAP plates containing the appropriate antibiotic or selection agent.

2.5. Materials for Biolistic Bombardment

1. Gold particles (0.6 μm ; Bio-Rad).
2. Ethanol (100%).
3. Spermidine (free base, 0.1 M in ddH₂O).
4. CaCl₂ stock solution (2.5 M in ddH₂O).
5. Sterile ddH₂O.
6. TAP plates without selection agent and plates containing the appropriate antibiotic or selection agent.
7. Plasmid DNA (20 μg for ten shots).
8. Macro-carrier disks and rupture disks (one of each per shot).
9. Particle gun (e.g., Bio-Rad PDS1000He) including stopping grid and vacuum pump.

2.6. Materials for Preparation of Autolysin

1. TAP-N medium.
2. Sterile filter (0.2- to 0.45- μm pore size).

2.7. Materials for Luciferase Activity Assay

1. Coelenterazine (P.J.K. GmbH, Kleinblittersdorf, Germany) substrate stock solution (1 mM in 100% ethanol; store at -20°C and avoid exposure to light).
2. Sample buffer [1.5 mM Tris-HCl (pH 7.8), 1 mM EDTA].
3. 96-Well white microtiter plates (nuncTM).
4. Assay buffer [0.1 M K₂HPO₄ (pH 7.6), 0.5 M NaCl, 1 mM EDTA].
5. Luminometer (MicroBeta[®] TriLux, Perkin-Elmer, Waltham, MA, USA).

2.8. Selectable Marker Genes

Currently available selectable marker genes for nuclear transformation of *Chlamydomonas* are listed in Table 1. In addition to antibiotic resistance genes, also genes encoding enzymes that complement metabolic deficiencies of auxotrophic mutant strains have been developed as selectable marker genes (Table 1). The optimum antibiotic concentration for selection is often somewhat strain dependent. It is, therefore, highly recommended to determine the sensitivity of the chosen recipient strain to the antibiotic prior to performing any transformation experiments.

2.9. Reporter Genes

The *C. reinhardtii* nuclear genome is unusually rich in GC (with 64% GC; see ref. 7), and therefore, the codon usage in *Chlamydomonas* differs substantially from that in higher plants. Codon optimization and chemical resynthesis of coding regions has greatly helped with the construction of reporter genes for *Chlamydomonas* (16). Some currently available reporters are listed in Table 2. It should be noted that fluorescent reporters (GFP and YFP) often do not work very well in *Chlamydomonas* unless dedicated expression strains are used (24, 25).

Table 1
Commonly used selectable marker genes for nuclear transformation
in *Chlamydomonas*

Marker gene	Gene product	Selection agent	Suggested concentration	Reference
<i>APHVIII</i>	Aminoglycoside 3'-phosphotransferase	Paromomycin (Sigma-Aldrich)	10 µg/ml	(19)
<i>CRYI_1</i>	Mutant version of S14 (ribosomal protein14 of the small subunit of the cytosolic ribosome)	Emetine (Sigma-Aldrich)	25 µg/ml	(20)
<i>BLE</i>	Zeocin-binding protein	Zeocin (Bio-Rad)	1.1 µg/ml (strain dependent)	(21)
<i>APH7"</i>	Aminoglycoside phosphotransferase 7"	Hygromycin (Roche)	10 µg/ml	(22)
<i>ARG7</i>	Argininosuccinate lyase	Nonsupplemented (i.e., arginine-free) growth medium	–	(23)
<i>NIT1</i>	Nitrate reductase	Medium with nitrate as sole nitrogen source	–	(6)

Table 2
Reporter genes for *Chlamydomonas*

Reporter gene	Gene product	Detection method	Reference
<i>GFP</i>	Green fluorescent protein	Fluorescence microscopy	(16, 25)
<i>YFP</i>	Yellow fluorescent protein	Fluorescence microscopy	(25)
<i>R-Luc</i> (<i>Renilla reniformis</i> luciferase)	Luciferase	Luminescence detector	(26)
<i>G-Luc</i> (<i>Gaussia princeps</i> luciferase)	Luciferase	Luminescence detector	(27)

2.10. Expression Elements

The choice of a suitable promoter is crucial to successful transgene expression in *Chlamydomonas*. For largely unknown reasons, most endogenous and foreign promoters result in poor transgene expression levels. The following four promoters have been reported to alleviate this problem and confer good transcriptional activity also in a transgenic context:

1. *PsaD* promoter (photosystem I protein subunit; see ref. 28).
2. *RBCS2* (small subunit of the ribulose biphosphate carboxylase/oxygenase) promoter and first intron of the *RBCS2* coding region (21).

- 3. *HSP70A/RBCS2* (+ *RBCS2 intron 1*) tandem promoter (heat shock protein 70A; see refs. 17, 21).
- 4. *HSP70A/HSP70B* heat shock-inducible tandem promoter (17).

The choice of the terminator region appears to be less critical and may not have a strong influence on transgene expression levels. Several terminators have been described (e.g., see refs. 17, 21, 28), and none of them seem to have particular advantages or disadvantages.

2.11. Algal Strains and Recommended Transformation Methods

Three different basic types of algal strains are available for transformation in *C. reinhardtii*: (1) cell wall-containing strains, (2) cell wall-deficient strains, and (3) expression strains (which are also cell wall deficient). Examples for each type of strains are given in Table 3. Cell wall-deficient strains have the advantage of high attainable transformation rates with simple transformation methods (glass bead transformation; Table 3). Their disadvantage is that the cell wall deficiency is usually accompanied by a loss in motility (due to underdeveloped, stumpy flagella), which in turn greatly hampers mating. Thus, these strains are notoriously difficult to cross, making genetic analyses very difficult. Cell wall-containing strains do not have these problems but are more difficult to transform, require more sophisticated DNA delivery techniques (Table 3), and often also give lower transformation rates. The recently generated expression strains UVM4 and UVM11 facilitate high-level transgene expression (and are particularly useful for sub-cellular localization analyses with the fluorescent markers YFP and GFP; see refs. 24, 25) and have very high transformation rates but are currently only available as cell wall-deficient strains (Table 3).

Table 3
Selected *Chlamydomonas* strains and their properties

Strain	Salient characteristics	Preferred transformation method
CC-621	Cell wall-containing strain; mt- (subclone of wild-type strain 137c, selected for high mating efficiency)	Autolysin-assisted glass bead transformation (see Subheading 3.2) or biolistic transformation (see Subheading 3.3)
cw15-302	Cell wall-deficient strain; arginine prototrophic (mt+, arg7)	Glass bead transformation (see Subheading 3.1)
Elow47	Cell wall-deficient strain; <i>CRT1-1/ARG7</i> cotransformant of cw15-302; see ref. 25	Glass bead transformation (see Subheading 3.1)
UVM4; UVM11	Cell wall-deficient strains; UV light-induced mutants of Elow47; see ref. 25	Glass bead transformation (see Subheading 3.1)

3. Transformation Methods

Largely for historical reasons, foreign genes are often introduced into the *Chlamydomonas* nuclear genome by cotransformation with a selectable marker gene and transgene of interest residing in two different plasmids. Cotransformation is quite efficient in *Chlamydomonas* with cointegration rates typically being in the range of 10–60% (6). However, apart from the somewhat simpler vector construction, there is no particular advantage associated with cotransformation, and accommodating marker gene and gene of interest in one and the same plasmid vector (25) represents a viable alternative.

The protocols described below represent two frequently used transformation methods in *Chlamydomonas*. A number of alternative transformation procedures have been developed (including, e.g., electroporation) but are somewhat less commonly used and, therefore, are not covered here.

3.1. Nuclear Transformation with the Glass Bead Method

This protocol is suitable for all cell wall-deficient strains. For *Chlamydomonas* wild-type strains that contain an intact cell wall, either autolysin-assisted glass bead transformation (see Subheading 3.2) or transformation via particle bombardment (see Subheading 3.3) should be performed.

Cultivate *Chlamydomonas* cells in liquid TAP medium under standard conditions and grow them to a density of $2\text{--}5 \times 10^6$ cells/ml. The plasmid DNA to be used for transformation should be linearized (see Note 2). In a standard transformation experiment, 1×10^8 cells are transformed with 0.2–1 μg DNA. For each transformation, fill approximately 300 mg acid-washed glass beads in a 2-ml reaction tube and sterilize the beads by autoclaving.

1. Harvest cells by centrifugation ($1,100 \times g$, 5 min) and resuspend the cell pellet in TAP medium to a cell density of 3.3×10^8 cells/ml.
2. Add 1 μg linearized plasmid DNA in a volume of 5 μl to the glass bead-containing reaction tube (place it above the glass beads at the wall of the reaction tube).
3. Add 303 μl (corresponding to 1×10^8 cells) of the cell suspension to the tube and vortex vigorously at maximum power for 15 s.
4. For a short regeneration round in the absence of selection, transfer cells to fresh TAP liquid medium (5–10 ml per transformation) and incubate under standard conditions for approximately 6 h.
5. Finally, harvest the cells by centrifugation, resuspend them in a smaller volume, and spread cells on plates with selection medium.

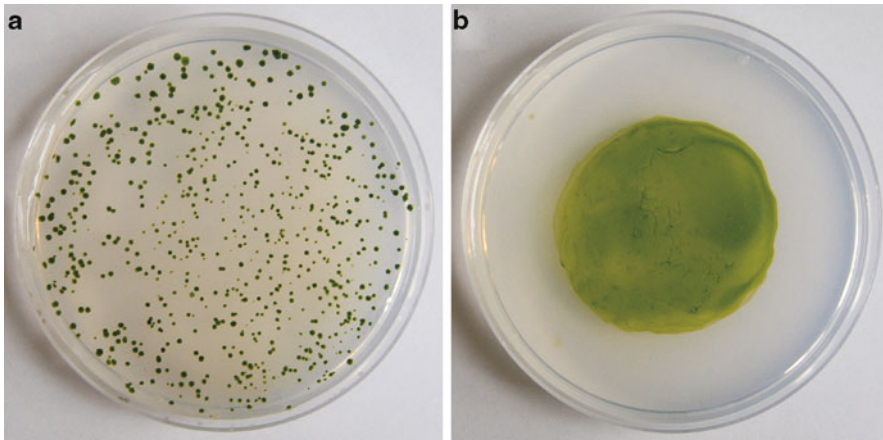


Fig. 1. Nuclear transformation in the model alga *Chlamydomonas reinhardtii*. (a) Selection of transgenic clones after glass bead-assisted transformation. The colonies shown here were selected for hygromycin resistance conferred by the *APH7* gene. (b) Sample preparation for biolistic transformation.

6. Incubate plates at standard conditions for approximately 2 weeks (see Note 3). Transgenic clones appear as growing green colonies (Fig. 1a).

3.2. Autolysin-Assisted Transformation of Cell Wall-Containing Strains

3.2.1. Autolysin Preparation

To facilitate transformation of cell wall-containing *Chlamydomonas* strains, the cell wall can be removed by autolysin treatment (29). Autolysin is a cell wall-digesting enzyme secreted by *Chlamydomonas* gametes during mating.

For autolysin preparation, choose a pair of highly mating-competent strains with opposite mating types (e.g., CC620mt+ and CC621mt-). Both strains can be obtained from the *Chlamydomonas* Center culture collection (<http://www.chlamy.org/strains.html>).

1. Grow both mating types under standard conditions in 250 ml liquid TAP medium to a final cell density of 1×10^7 cells/ml and harvest cells by centrifugation ($1,100 \times g$, 5 min).
2. Discard the supernatant, wash the cells with TAP-N medium, and collect them again by centrifugation ($1,100 \times g$, 5 min).
3. Carefully resuspend the cell pellet in TAP-N medium to a final density of 2×10^6 cells/ml.
4. Incubate the culture under continuous light and shaking overnight at 22–24°C (see Note 4).
5. Harvest gametes by centrifugation ($1,100 \times g$, 5 min) and resuspend the cell pellet in TAP-N medium to a cell density of 2×10^7 cells/ml.
6. To allow mating, mix the cells of the two mating types in a 1:1 ratio and incubate for 1–2 h at 22–24°C under bright light, without shaking.

7. Centrifuge cell mixture ($2,000\times g$, 5 min) and transfer the supernatant to a new centrifugation tube. A second centrifugation step at $20,000\times g$ for 15 min is required to remove residual cell debris.
8. Finally, filter-sterilize the solution using a 0.2- to 0.45- μm pore size filter and store it in aliquots at -20°C .

3.2.2. Autolysin Treatment and Transformation of Cell Wall-Containing Strains

Prior to transformation, the cell culture (3.3×10^6 cells/ml) is incubated with the same volume of autolysin solution for 1 h (at $22\text{--}24^{\circ}\text{C}$ with gentle shaking). Another deviation from the standard glass bead transformation protocol is the addition of 100 μl sterile 20% polyethylene glycol (PEG; M_r 6000) to each transformation tube prior to vortexing. PEG acts as an osmotic protectant to the autolysin-treated cells. Apart from these two differences, the transformation protocol is identical to the standard glass bead transformation protocol (see Subheading 3.1).

3.3. Biolistic Transformation with a Particle Gun

3.3.1. Gold Particle Preparation for Biolistic Transformation

All steps should be performed on ice or in the cold room (at 4°C). Water and 100% ethanol must be ice-cold.

1. Weigh 2 mg gold particles per ten shots and wash them in 600 μl 100% EtOH by vortexing for at least 1 min at maximum power.
2. Centrifuge <1 s at $1,700\times g$ in a microfuge and remove the supernatant completely.
3. Resuspend the particles in 600 μl sterile ddH_2O (see Note 5), centrifuge ($1,700\times g$, <1 s), and discard the supernatant.
4. Resuspend the particles very carefully in 250 μl sterile ddH_2O .
5. For ten shots, take 245 μl of the gold suspension and add 10 μg DNA (concentration $1\text{--}2\text{ }\mu\text{g}/\text{ml}$), 250 μl 2.5 M CaCl_2 , and 50 μl spermidine in the given order. (Vortex briefly immediately after addition of each component).
6. Incubate on ice for 10 min with brief vortexing every minute.
7. Centrifuge <1 s at $800\times g$ in a microfuge and remove the supernatant completely.
8. Add 600 μl of 100% EtOH and carefully resuspend the particles by pipetting and vigorous vortexing.
9. Centrifuge <1 s at $800\times g$ in a microfuge and remove the supernatant completely.
10. Repeat steps 8 and 9.
11. Resuspend the particles very carefully in 65 μl 100% EtOH by pipetting and vigorous vortexing.
12. Use 6.5 μl per shot and carefully resuspend particles immediately before use.

3.3.2. Biolistic Bombardment and Selection of Transgenic Clones

1. Grow *Chlamydomonas* cells in at least 200 ml liquid TAP medium under standard conditions to a final cell density of $2\text{--}5 \times 10^6$ cells/ml.
2. Harvest cells by centrifugation ($1,100 \times g$, 5 min) and for each shot, spread 4×10^7 cells on a TAP plate lacking any selection agent. (It is recommended to spread the cells in the center of the Petri dish as a circle with a diameter of 4–5 cm; see Fig. 1b).
3. Let the cell suspension dry on the TAP plate for a few minutes in the sterile bench and, during this time, start with the preparation of the gold particles (see Subheading 3.3.1).
4. Sterilize the equipment of the particle gun (macro-carrier disks, rupture disks, stopping screens, flying disk assembly, and Petri dish holder) using 100% EtOH and let the parts dry under sterile conditions.
5. Bombard the cell lawn on the plates with the DNA-coated gold particles using a particle gun (e.g., the Bio-Rad PDS1000He biolistic gun) according to the instruction of the manufacturer (for more detailed information, see also ref. 30).
6. After bombardment, close the plates and incubate them overnight in the dark at $22\text{--}24^\circ\text{C}$.
7. Finally, wash the cells from the TAP plate with liquid TAP medium, transfer them to a sterile 10- to 12-ml centrifuge tube, and sediment the cells by centrifugation ($1,100 \times g$, 5 min).
8. Resuspend the cells in 300–500 μl TAP, spread them on a TAP plate containing the appropriate selection medium, and incubate the plates under standard conditions. Clones growing in the presence of the selection agent typically appear after 2–3 weeks.

3.4. Analysis of Transgenic Strains

Selected antibiotic-resistant (or prototrophic) clones are transferred to liquid medium, grown under continuous selection, and analyzed using standard molecular methods (Southern blotting, PCR, northern blotting, and western blotting). Biolistic transformation often results in integration of multiple copies of the transformation construct, and therefore, determining transgene copy numbers by Southern blotting is highly recommended.

Expression and subcellular localization of the GFP and YFP reporters can be analyzed by confocal laser-scanning microscopy (e.g., TCS SP2; Leica, <http://www.leica-microsystems.com>) using an argon laser for excitation (at 488 nm for GFP and 514 nm for YFP), a 490- to 510-nm filter for detection of GFP fluorescence, a 510- to 535-nm filter for detection of YFP fluorescence, and a 630- to 720-nm filter for detection of chlorophyll fluorescence (24, 25). For subcellular localization analyses, it is recommended

to use YFP rather than GFP (whose detection suffers from relatively high background fluorescence in *Chlamydomonas*) and the expression strains UVM4 or UVM11 (Table 3; see refs. 24, 25).

The synthetic *G-Luc* gene provides a highly sensitive bioluminescent reporter of gene expression (27). Expression can be easily monitored and quantitated by measuring luciferase activity in a luminometer (see Subheading 3.4.1).

3.4.1. Luciferase Activity Assay

Cultivate *Chlamydomonas* cells in liquid TAP medium under standard conditions to a density of $3\text{--}6 \times 10^6$ cells/ml.

1. Harvest 500 μl of the cell culture by centrifugation ($2,300 \times g$, 3 min at 4°C) and resuspend the cells in the same volume of sample buffer.
2. Freeze samples at -20°C for at least 20 min or alternatively store them at -20°C .
3. Thaw samples and transfer 20 μl to a 96-well white microtiter plate. Add 125 μl assay buffer to each well and incubate at room temperature for 15 min.
4. During the 15-min incubation step, prepare the substrate solution by diluting the 1 mM coelenterazine stock solution to a final concentration of 0.01 mM in assay buffer (see Note 6).
5. Add 50 μl substrate solution to each well and measure bioluminescence using a luminometer with autoinjection system. Normalize for background luminescence by measuring wells containing only buffer and/or buffer with cells lacking the *Luc* gene. All data (given in luminescence counts per second; LCPS) should be normalized to either cell number or chlorophyll content.

4. Notes

1. For preparation of Hutner's trace metals, dissolve EDTA separately in 250 ml ddH_2O while heating. Dissolve all other components in the given order in 550 ml ddH_2O while heating (up to 100°C). Then add the hot EDTA solution to the boiling mixture. Cool down the (dark-green) mixture to 70°C and adjust the pH to 6.5–6.8 with KOH (20% w/v) at 70°C . Add distilled water to a final volume of 1 L and incubate solution in the dark at room temperature under moderate shaking (to allow aeration) for 2 weeks, during which time it should turn purple. After filtration, the trace metal stock solution can be stored at 4°C .
2. Plasmid DNA is linearized using a restriction enzyme that cuts only once in the vector backbone. For cotransformation, add

0.2–1 µg DNA (in a volume of 5 µl) of each plasmid per reaction tube and omit mixing prior to addition of the cells. For maximum cotransformation efficiency, a 3:1 molar ratio of the vector containing the transgene of interest and the vector carrying the selectable marker gene is recommended.

3. Consider that for some light-sensitive selection agents (e.g., Zeocin), it may be better to first incubate the plates overnight in the dark and then for 2 weeks under low-light conditions (5 µmol/m²/s).
4. Gametes are formed under nitrogen deprivation conditions (in TAP-N).
5. DNA-coated gold particles tend to aggregate. Careful resuspension of the particles is crucial to the success of the transformation experiment. Clumpy particles hit fewer target cells and will cause more lethal cell damage upon penetration.
6. Always prepare the substrate solution immediately before use and avoid exposure to light.

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Part III

Transformation Technology: Rice

A High-Efficiency *Agrobacterium*-Mediated Transformation System of Rice (*Oryza sativa* L.)

Kenjiro Ozawa

Abstract

Agrobacterium-mediated transformation of rice has been routinely performed according to the protocol reported by Hiei et al. (Plant J. 6:271–282, 1994). However, several elite *japonica* and many *indica* varieties cannot be efficiently transformed by *Agrobacterium* system. Also a large number of transformants are required to generate T-DNA insertion and FOX libraries as well as gene-targeting studies. To overcome these challenges, we established a high-efficiency transformation system in rice by cocultivating rice calli with *Agrobacterium* on filter papers moistened with enriched liquid media instead of using solid media (Ozawa, Plant Sci. 176:522–527, 2009; Ozawa and Takaiwa, Plant Sci. 179:333–337, 2010). In this system, the transformation efficiency of the calli is almost 100% in many varieties.

Key words: Rice, *Agrobacterium*, Transformation, Liquid medium

1. Introduction

The transfer of T-DNA and its integration into the plant genome is influenced by numerous factors, such as plant genotype, explants, strains of *Agrobacterium*, plasmid vectors, addition of vir-gene-inducing synthetic phenolic compounds, selection marker, and various conditions of tissue culture. In tissue culture systems, selection of actively growing regenerable calli is one of the most important factors for efficient plant transformation. Also, optimization of culture conditions for cocultivation of rice calli with *Agrobacterium* is important (1–3). The addition of acetosyringone in cocultivation media markedly increases the efficiency of transformation in rice (1). On the other hand, excessively high bacteria concentration lowers the overall transformation efficiency. Another factor we demonstrated that lowers transformation efficiency is the production of an unidentified compound produced by rice calli during *Agrobacterium*-mediated transformation. The compound easily

diffuses into medium, lowering the transformation efficiency. However, these two factors are easily controlled by cocultivating rice calli with *Agrobacterium* on filter papers moistened with small amounts of liquid media (2, 3). Compared with the transformation efficiency of calli cocultivated on solid media, transformation efficiency is effectively increased by using the filter paper method for many varieties of rice, including those that previously yielded very low transformation rates.

2. Materials

All media (Table 1) are autoclaved for 12 min at 120°C and prepared in 90 mm×20 mm plastic Petri dishes. Sterilized appropriate selective agents, L-cysteine and acetosyringone, are added to media after autoclaving.

1. Meropen (Dainippon Sumitomo Pharma, Japan) is dissolved at 25 mg/mL in water, filter-sterilized, and stored in single-use aliquots at –20°C.
2. Hygromycin B solution (50 mg/mL) (Wako, Japan).
3. Kanamycin is dissolved at 50 mg/mL in water, filter-sterilized, and stored in single-use aliquots at –20°C.
4. Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) (Aldrich, USA) is dissolved at 150 mg/mL in DMSO and stored at –20°C.
5. L-Cysteine is dissolved at 10 mg/mL in water, filter-sterilized, and stored in single-use aliquots at –20°C.
6. Plastic Petri dishes (90 mm×20 mm).
7. Sterile filter paper (9 cm in diameter, No. 2, Advantec, Japan, comparable to Whatman Grade No. 2 filter paper).
8. Parafilm (Pechiney Plastic Packaging Company, USA).
9. Surgical tape (Surgical tape-21N, Nichiban Co., Ltd., Japan).
10. 25 and 30°C growth rooms.
11. R-N6D medium (4), 50 mL in Petri dish (callus induction medium for all varieties).
12. R-DKND medium (5), 50 mL in Petri dish (callus propagation medium for elite *japonica* varieties).
13. R-N6AS medium (*Agrobacterium*-infection and cocultivation medium for easily cultured varieties).
14. R-DKNAS medium (*Agrobacterium*-infection and cocultivation medium for elite *japonica* varieties).

Table 1
Media used for tissue culture and *Agrobacterium*-mediated transformation

	R-N6D	R-N6AS	R-MSReg	R-DKND	R-DKNAS	R-DKNReg	R-MSHF	AB
Macroelement (mg/L)								
KNO ₃	2,830	2,830	1,900	800	800	800	1,900	
NH ₄ NO ₃			1,650				1,650	
(NH ₄) ₂ SO ₄	463	463		67	67	67		
MgSO ₄ ·7H ₂ O	185	185	370	250	250	250	370	296
CaCl ₂ ·2H ₂ O	166	166	440	150	150	150	440	10
KH ₂ PO ₄	400	400	170				170	
NaH ₂ PO ₄ ·2H ₂ O				355	355	355		1,300
NH ₄ Cl								1,000
K ₂ HPO ₄								2,950
KCl								150
Micro element (mg/L)								
EDTA·2Na	37.3	37.3	37.3	7.5	7.5	7.5	37.3	
FeSO ₄ ·7H ₂ O	27.8	27.8	27.8	5.5	5.5	5.5	27.8	
MnSO ₄ ·4·6H ₂ O	4.4	4.4	22.3	1.6	1.6	1.6	22.3	
ZnSO ₄ ·7H ₂ O	1.5	1.5	8.6	2.2	2.2	2.2	8.6	
KI	0.8	0.8	0.83				0.83	
H ₃ BO ₃	1.6	1.6	6.2	3	3	3	6.2	
Na ₂ MoO ₄ ·2H ₂ O	0.01	0.01	0.25	0.125	0.125	0.125	0.25	
CuSO ₄ ·5H ₂ O	0.01	0.01	0.025	0.125	0.125	0.125	0.025	
CoCl ₂ ·6H ₂ O	0.01	0.01	0.025				0.025	
Organic elements (mg/L)								
Glycine	2	2	2	2	2	2	2	
Nicotinic acid	1	1	1	1	1	1	1	
Pyridoxine HCl	1	1	1	1	1	1	1	
Thiamine HCl	10	10	10	10	10	10	10	
L-Proline	1,150	1,150		1,150	1,150			
L-Glutamine				731	731	731		
L-Aspartic acid				666	666	666		
L-cystein		100			100			
Casamino acids	300	300	2,000	300	300	2,000		
Phytohormones (mg/L)								
2,4-D	2	2		2	2			
NAA			0.1			0.1		
Kinetin			2.5			2.5		
Acetosyringone		15			15			
Carbon source (g/L)								
Sucrose	30	30	30	30	30	30	30	
Sorbitol			30			30		
Glucose		5			5			5
Gelling agents (g/L)								
Gelrite	3		4	3		4	3	
Agar								15
pH	5.7	5.4	5.7	5.7	5.4	5.7	5.7	7

All media are autoclaved for 12 min at 120°C and prepared in 90 mm × 20-mm plastic Petri dishes. Appropriate sterilized selective agents, L-cysteine and acetosyringone, were added to media after autoclaving

15. R-N6D medium supplemented with Meropen (25 mg/L) and hygromycin (50 mg/L) or appropriate selective agents (R-N6Sel), 50 mL in Petri dish (selection medium for easily cultured varieties).
16. R-DKND medium supplemented with Meropen (25 mg/L) and hygromycin (50 mg/L) or appropriate selective agents (R-DKNSel), 50 mL in Petri dish (selection medium for elite *japonica* varieties).
17. R-MSReg medium (6) supplemented with Meropen (25 mg/L) and hygromycin B (50 mg/L) or appropriate selective agents, 50 mL in Petri dish (regeneration medium for easily cultured varieties) (see Note 1).
18. R-DKNReg medium supplemented with Meropen (25 mg/L) and hygromycin B (50 mg/L) or appropriate selective agents, 50 mL in Petri dish (regeneration medium for elite *japonica* varieties) (see Note 1).
19. R-MSHF medium, 50 mL in Petri dish.
20. AB medium supplemented with kanamycin (50 mg/L) or suitable antibiotics, 30 mL in 9-cm Petri dish.

3. Methods

Agrobacterium strains EHA101, EHA105, and LBA4404, harboring binary vectors such as pIG121Hm and pCAMBIA1301 containing the *hpt* gene, are used in many laboratories (7, 8) (see Note 2). This protocol is based on the one published by Ozawa (2). All procedures must be carried out under sterile conditions. Actively growing calli are induced from many easily cultured varieties (Nipponbare, Kitaake, Dular, Yukihihikari, Hoshinoyume, etc.) on N6 media. However, elite *japonica* rice (Koshihikari and related cultivars, Haenuki, Hitomebore, Akitakomachi, etc.) is known for its poor responses in tissue culture on N6 media, while showing good responses in tissue culture on DKN media in which the main nitrogen sources are amino acid. So after the induction of calli on N6 media, they are cultured on DKN media during all culture processes (2). This procedure can be optimized also for easily cultured varieties and elite *japonica* rice varieties. Many *indica* varieties may require optimization of the culture conditions, which should be done before transformation.

3.1. Callus Induction and Propagation

1. Dehusk the rice seed carefully to avoid injury of the pericarp. Sterilize in 10% sodium hypochlorite by gently shaking (20–30 rpm) for 40 min (see Note 3).

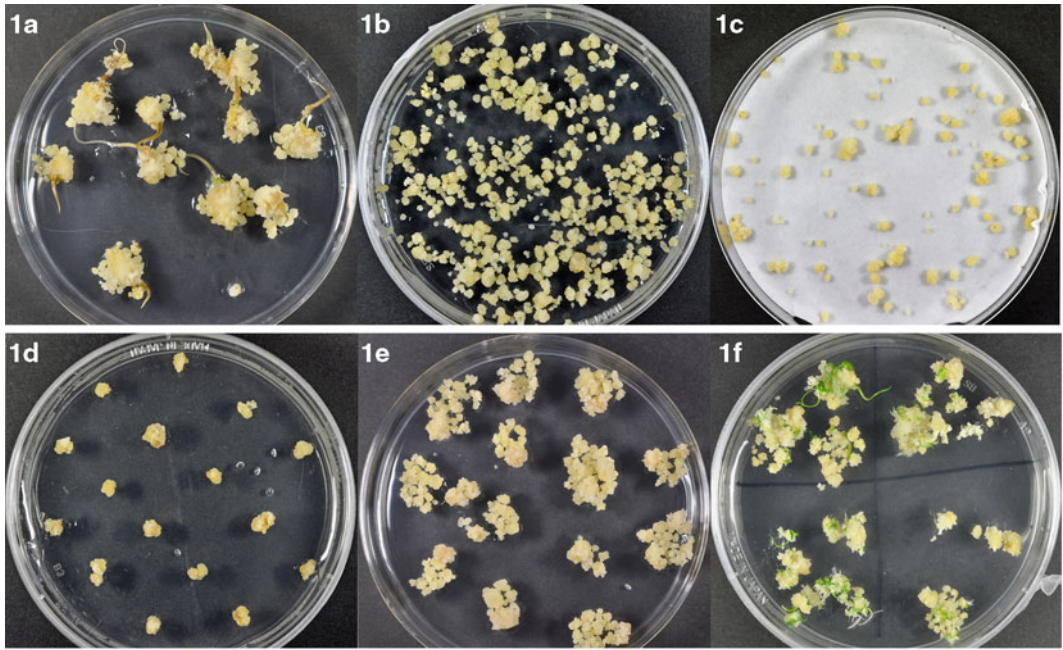


Fig. 1. Process of *Agrobacterium*-mediated transformation. (a) Seeds (cv; Nipponbare) were cultured for 3 weeks on R-N6D medium. (b) Vigorously dividing calli were subcultured on the same fresh medium for 3 days. (c) Calli cocultured on three pieces of filter paper moistened with 3.6 mL of R-N6AS medium for 3 days at 25°C. (d) Cocultured calli on liquid medium were subjected to hygromycin selection for 10 days. (e) Calli were subjected to second hygromycin selection for 14 days. (f) Hm-resistant calli were cultured on regeneration medium for 3 weeks.

2. Pour off the sodium hypochlorite solution. After three rinses with sterilized water, put the seeds on filter paper.
3. Using the forceps, transfer about 10–20 seeds on solid R-N6D medium. Seal the plates with surgical tape (see Note 4) and incubate (30°C, 16-h days).
4. This step is needed only for elite *japonica* varieties. Small differentiated tissues are detected at the scutellum tissue of seed after 1 week of culture on R-N6D media. Transfer these seeds on solid R-DKND medium. Seal the plates with surgical tape and incubate (30°C, 16-h days).
5. After about 3 weeks (Fig. 1a), subculture vigorously dividing calli on the same fresh medium for 3 days (Fig. 1b), and then use the calli for transformation (see Note 5).

3.2. *Agrobacterium* Infection and Cocultivation

1. Culture *Agrobacterium* harboring binary vector on AB medium supplemented with kanamycin (50 mg/L) or appropriate antibiotics for 3 days at 28°C in the dark.
2. Collect a small amount of bacteria from the plate and suspend in liquid R-N6AS or R-DKNAS medium in 50-mL centrifuge tube. For *Agrobacterium* infection, adjust the density of the bacteria ($OD_{600} = 0.05\text{--}0.2$).

3. For infection, immerse the calli directly in a bacterial suspension for 2 min.
4. After infection, remove excess bacteria by blotting calli on filter paper. Transfer the calli onto two pieces of sterilized filter paper placed in a 9-cm-diameter Petri dish containing 3.6 mL of liquid R-N6AS or R-DKNAS medium (see Note 6).
5. Seal the plates with Parafilm to prevent evaporation of the media and cocultivate for 3 days at 25°C in the dark.

3.3. Selection for Transformed Calli

1. After 3 days of cocultivation (Fig. 1c), wash the calli once in sterile water containing Meropen (50 mg/L) to remove *Agrobacterium*. Blot the cocultured calli on a filter paper (see Note 7).
2. Using the forceps, transfer about 20 calli on solid R-N6Sel or R-DKNSel medium. Seal the plates with surgical tape and incubate (30°C, 16-h days).
3. After about 10 days (Fig. 1d), transfer all calli on the same fresh medium. Seal the plates with surgical tape and incubate (30°C, 16-h days). Hm-resistant calli begin to appear after 10–14 days on second selection (Fig. 1e) (see Note 8). The actively growing calli are easily distinguishable from nontransformed calli.

3.4. Regeneration

1. Using the forceps, transfer 12 calli which are actively growing on R-MSReg or R-DKReg medium. Seal the plates with Parafilm and incubate (30°C, 16-h days).
2. After about 3 weeks (Fig. 1f), transfer regenerated shoots on R-MSHF medium containing Meropen (25 mg/L). Seal the plates with surgical tape and incubate (30°C, 16-h days) before potting.

4. Notes

1. Dry plates for 2–3 days at room temperature or 30 min with the lids slightly off at room temperature in a laminar flow hood.
2. Most effective selection marker is hygromycin phosphotransferase (*hpt*) in rice, which confers resistance to the antibiotic hygromycin (30–100 mg/L). So, *Agrobacterium* (EHA101, EHA105, and LBA4404 are used in many laboratories) harboring binary vectors such as pIG121Hm or pCAMBIA1301 containing the *hpt* gene are recommended. Phosphinothricin acetyl transferase (*bar*), mutated acetolactate synthase (W548L/S627I mutated *ALS* gene from rice) genes that confer resistance to the herbicides bialaphos (2–5 mg/L) and bispyribac-sodium (0.25–2.5 µM), respectively, are also used to select transformed

- calli in rice (7, 9). Compared with hygromycin as selective agent, transformation efficiency of these selective agents is low.
3. Seeds preserved for more than 1 year after harvesting are preferable. Seeds used immediately after harvest are not suitable for the induction of calli.
 4. Evaporation of a large amount of the medium in plastic Petri dishes frequently occurs when surgical tape is used as sealing material, which results in poor growth of calli.
 5. If calli are growing well, this step may be omitted.
 6. The amount of the cocultivation media is the most important factor for efficient transformation. If transformation efficiency is low, the amount of the cocultivation media should be adjusted from 3.3 to 3.8 mL.
 7. For easily cultured varieties (Nipponbare, Kitaake, Dular, Yukihihikari, Hoshinoyume, etc.), this step may be omitted. *Agrobacterium* overgrowth is usually not observed in this cocultivation condition.
 8. If *bar* or *ALS* genes are used as the selection marker, resistant calli usually begin to appear within 3–5 weeks after selection, so another subculture should be necessary.

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Selection of Transgenic Rice Plants Using a Herbicide Tolerant Form of the Acetolactate Synthase Gene

Masaki Endo, Tsutomu Shimizu, and Seiichi Toki

Abstract

Acetolactate synthase (ALS) is an enzyme in the biosynthetic pathway for branched-chain amino acids, and bispyribac-sodium (BS), a pyrimidinyl carboxy herbicide, is a well-known inhibitor of ALS activity. However, it appears that a mutated form of rice *ALS* [*OsmALS* (W548L/S627I)] confers resistance to BS. We succeeded in using *OsmALS* with native *OsALS* promoter and terminator region for a selection marker of rice transformation. Because this selection marker cassette is originally from the rice endogenous genome, it can be expected to be publicly acceptable.

Key words: *Agrobacterium*-mediated transformation, Acetolactate synthase, Rice (*Oryza sativa* L.), Herbicide resistance, Bispyribac-sodium

1. Introduction

Some consumers oppose the use of transgenic plants containing selectable marker genes, because these genes are derived from bacteria. In order to minimize their concerns, trials to remove selectable marker genes from transgenic plants have been performed. For example, the MAT vector system was developed as a method to remove a selectable marker (1, 2), and cotransformation has also been used to remove a selectable marker gene (3, 4), but these complicated processes are sometimes troublesome for practical use in the production of transgenic plants.

An alternative method to solve these problems, using a marker gene derived from safely edible plants, has been proposed. Shimizu et al. (5) isolated a two-point mutated gene of acetolactate synthase (ALS) from herbicide-resistant rice callus and showed that overexpression of this mutated *OsALS* by cauliflower mosaic virus 35S promoter conferred bispyribac-sodium (BS) resistance

to rice plants. The mutations in *OsALS* involve the residues of tryptophan (TGG) to leucine (TTG) at amino acid 548 (W548L) and serine (AGT) to isoleucine (ATT) at amino acid 627 (S627I). ALS is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids isoleucine, leucine, and valine. ALS is the target site of sulfonylurea (SU), imidazolinone (IMI), and pyrimidinyl carboxy (PC) herbicides (6), and several mutations in ALS genes that confer herbicide resistance in several plant species are known (7). The mutated *ALS* gene coupled with ALS-targeting herbicides has been applied to a trial for the production of transgenic plants. We developed a publicly acceptable new selectable marker gene cassette using *OsALS* with W548L and S627I mutations (*OsmALS*) (8). In this work, we cloned a genomic DNA fragment containing regulatory and coding sequences of *OsALS* and mutagenized the *ALS* gene into a herbicide-resistant form (*OsmALS*). After transfer of this construct to the rice genome, transgenic plants were efficiently selected with BS. Here we present the strategy of rice transformation method using *OsmALS* as a selection marker.

2. Materials

2.1. *Agrobacterium*-Mediated Transformation Using Primary Callus

2.1.1. Plant and *Agrobacterium* Materials

1. Rice mature seeds (see Chapter 7 written by Saika et al.) (see Note 1).
2. *Agrobacterium tumefaciens* strain EHA105 (see Chapter 7 written by Saika et al.).

2.1.2. Binary Vector

The selectable marker functional cassette, the 5-kb fragment of rice genomic DNA containing the BS-resistant type of ALS (*OsmALS*), was cloned into the binary vector pPZP200 (9) (Fig. 1). To confer BS resistance, 2.2 kb of promoter region and 0.5 kb of terminator region of *OsALS* with W548L and S627I mutations are enough (8).

2.1.3. Media for *Agrobacterium*-Mediated Transformation (10)

All media are stored at 4°C. Autoclaved media are poured by 50 mL in each dish (9 cm in diameter and 2 cm in depth). Media are incubated at room temperature, and the lids are opened to dry up water drops on the surface of medium in a clean bench just before use.

1. N6D medium: see Chapter 7 written by Saika et al.
2. AB medium: see Chapter 7 written by Saika et al.

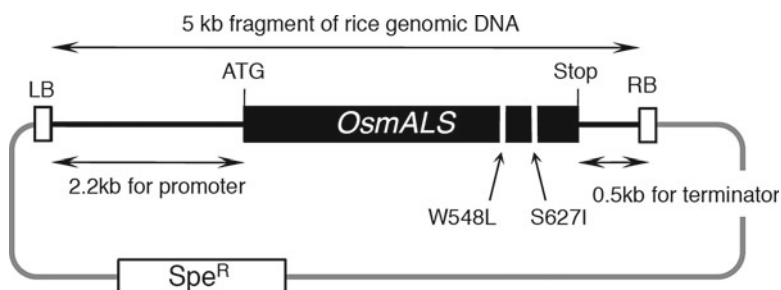


Fig. 1. Schematic representation of the *OsmALS* vector. The 5.0-kb fragment containing the BS-resistant type of ALS *OsmALS* was cloned into the binary vector pPZP200. The *black thick line* represents 5'- and 3'-noncoding region. The *black box* represents the coding region. *Black arrows* show two point mutations, W548L (TGG to TTG) and S627I (AGT to ATT), which give BS resistance. Both mutations generate recognition sites for the restriction enzyme *Mfe*I (CAATTG), which allows rapid detection of this transgene in rice genomic DNA.

3. AAM medium (liquid medium): see Chapter 7 written by Saika et al.
4. 2N6AS medium: see Chapter 7 written by Saika et al.

2.1.4. Stock Solution

1. 2,4-Dichlorophenoxy acetic acid (2,4-D): 5 mg/mL. Dissolve the powder in dimethyl sulfoxide (DMSO). Store at 4°C.
2. Acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone): 100 mg/ml. Dissolve the powder in DMSO. Store at 4°C.
3. Meropenem (Wako Pure Chemical Industries): 12.5 mg/mL. Dissolve the powder in hot ddH₂O. Store at 4°C (see Note 2 in Chapter 7 written by Saika et al.).

2.2. Selection and Regeneration of BS-Resistant Calli After Agrobacterium-Mediated Transformation

2.2.1. Media for Selection of BS-Resistant Calli and Regeneration of BS-Resistant Plantlets

1. Selection medium: after autoclaving N6D medium, add 1 mL of 12.5 mg/mL meropenem and 0.75 mL of 1 mM BS to 1 L of N6D medium. Final concentration of BS is 0.75 μ M.
2. Regeneration medium: 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, one bag of MS salts (Murashige and Skoog Plant Salt Mixture, Wako Pure Chemical Industries, Osaka, Japan), MS-vitamins (100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, and 0.1 mg/L thiamine HC), 0.02 mg/L NAA, 2 mg/L kinetin, and 4 g/L gelrite (Wako Pure Chemical Industries), pH 5.8. After autoclaving, add 1 mL of 12.5 mg/mL meropenem and 0.75 mL of 1 mM BS.
3. Hormone-free medium: 30 g/L sucrose, one bag of MS salts (Murashige and Skoog Plant Salt Mixture, Wako Pure Chemical Industries, Osaka, Japan) MS-vitamin (100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HC), and 4 g/L gelrite (Wako Pure Chemical Industries), pH 5.8. After autoclaving, add 1 mL of 12.5 mg/mL meropenem and 0.75 mL of 1 mM BS.

2.2.2. Stock Solutions

1. BS (CAS Number, 125401-92-5; MW=452.36) (Kumiai chemical Industry Co., Tokyo, Japan): 10 mM for long storage. Dissolve the powder in ddH₂O. Store at -20°C. For short storage, dilute 10 mM stock to 1 mM.
2. Kinetin: 0.2 mg/mL. Dissolve with small amount of 1 N KOH or 1 N NaOH, stir in water, and store at 4°C.
3. Naphthalene acetic acid (NAA): 0.2 mg/mL. Dissolve with small amount of 1 N KOH or 1 N NaOH, stir in water, and store at 4°C.

2.3. Confirmation of the Existence of OsmALS by PCR-Mfe I Digestion Analysis

2.3.1. DNA Extraction

DNeasy Plant Mini Kit (Qiagen, Germany).

2.3.2. Polymerase Chain Reaction

DNA polymerase, KOD Dash (Toyobo, Osaka, Japan) with 10× buffer and 2 mM dNTPs provided with enzyme. Primers: OsALS-30 5'-GCCGGTCTGGGACACCTCGATGAATCTA-3', reverse primer, OsALS-33 5'-GGAGAGTACTTCGTGTGATGACAGTTGAGCA-3'.

2.3.3. Mfe I Digestion

Restriction enzyme *Mfe* I (New England Biolabs Inc., MA).

3. Methods

3.1. Agrobacterium-Mediated Transformation Using Primary Callus

See Chapter 7 written by Saika et al.

3.2. Selection and Regeneration of BS-Resistant Calli After Agrobacterium-Mediated Transformation

1. Collect cocultivated calli into 50-mL tube. Wash the calli with sterilized water >7–8 times and subsequently wash with 12.5 ml/L meropenem solution >2 times to remove *Agrobacterium* growing on calli. Place washed calli onto the sterilized paper filter for exclusion of extra solution. Place nine pieces of calli onto the selection medium and grow at 33–36°C for 2 weeks.
2. Transfer calli from the first selection medium to the fresh selection medium containing 0.75 μM BS (see Note 2).
3. Transfer calli to fresh selection medium every 2 weeks until BS tolerant calli can be distinguished from BS sensitive non-transformed calli (Fig. 2a).

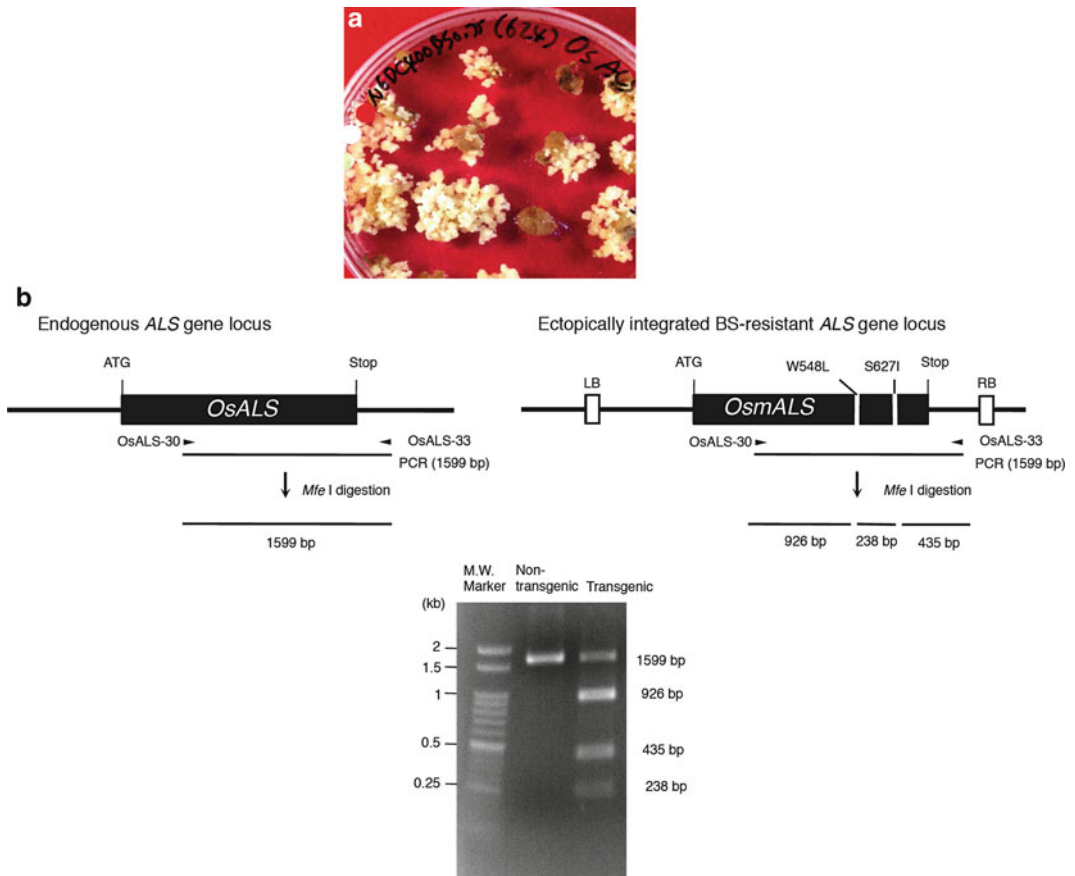


Fig. 2. Selection and confirmation of transgenic plants. (a) Transgenic rice calli selected on selection medium containing $0.75 \mu\text{M}$ BS. (b) Schematic representation of PCR-*Mfe* I digestion analysis. The *bottom panel* indicates typical results of genotyping assay with nontransgenic and transgenic plants.

4. For each original infected calli, transfer resistant embryogenic calli (white/yellow proliferative calli arising from the scutellum) onto regeneration medium. Incubate Petri dishes under continuous light for 1 week until plantlets arising from the calli. Cultivate at least six independent transgenic events (embryogenic calli arising from six different seedlings) per Petri dish.
5. Isolate plantlets arising from the calli, transfer onto hormone free medium, and incubate under a continuous light regime for 1 or 2 weeks in order to promote shoot and root development.

3.3. Confirmation of the Existence of *OsmALS* by PCR-*Mfe* I Digestion Analysis

BS-tolerant mutations can occur spontaneously, so we recommend checking the existence of *OsmALS* in BS-resistant regenerated plants by PCR-*Mfe* I digestion analysis (8). W548L and S627I mutations on *OsmALS* create novel recognition sites for the

restriction enzyme *Mfe* I (CAATTG). *Mfe* I digestion of polymerase chain reaction (PCR) products amplified using forward primer on upstream of W548L and reverse primer on downstream of S627I mutations can detect the existence of W548L and S627I mutation on *OsmALS* easily. The combination of the double mutation W548L/S627I in the rice genome was discovered only after 6 months of selection of BS-tolerant rice calli in liquid culture medium (5).

1. Prepare DNA from regenerated seedlings by using DNA extraction kit, DNeasy plant Mini Kit (Qiagen, Germany).
2. Perform PCR using OsALS-30 and OsALS-33 primers with 2.5 U of KOD Dash (Toyobo) as a thermostable DNA polymerase in 50 μ L or reaction mixture containing 10 nmol of each deoxyribonucleotide triphosphate, 10 pmol of forward and reverse primers, and 30 ng of genomic DNA. The thermal cycle program is as follows: 98°C for 2 min, 35 cycles of (98°C for 15 s and 68°C for 1.5 min), and 68°C for 7 min.
3. Digest PCR products (10 μ L from 50 μ L reaction mix) with 10 U of restriction enzyme *Mfe* I (New England Biolabs Inc., MA) at 37°C for 2 h.
4. Separate the PCR products digested with *Mfe* I and analyze on a 2% agarose gel. No *Mfe* I site exists in the endogenous *ALS* locus of the rice genome, but two *Mfe* I sites exist in *OsmALS* corresponding to W438L and S627I mutations. Thus, *Mfe* I digestion of PCR fragments yields 1,599-, 926-, 435-, and 238-bp band from PCR products amplified from genomic DNA from transgenic rice plants (Fig. 2b).

4. Notes

1. BS sensitivity of *indica* and *indica*-derived cultivars was relatively lower than the japonica cultivars in young seedlings (11). Because of this relatively lower BS sensitivity than japonica rice, the use of BS as a selectable reagent for transformation of *indica* and *indica*-derived cultivars is more difficult than in *japonica* cultivars. Though Taniguchi et al. (12) reported that the callus of *indica* and *indica*-derived cultivars showed high sensitivity to BS and they successfully obtained transgenic rice plants of *indica* and *indica*-derived cultivars by using BS for selection of transformed cells with *OsmALS* (W548L/S627I) as the selectable marker gene for *Agrobacterium*-mediated transformation, we recommend testing BS sensitivity of rice calli derived from seed of own cultivars before starting transformation.

2. BS selection is less critical than the selection using antibiotic-resistant genes and promoters derived from viruses such as hygromycin selection for hygromycin phosphotransferase or G418 selection for neomycin phosphotransferase driven by cauliflower mosaic 35S promoter. So BS selection sometimes needs one or two additional transfer to new selection medium than hygromycin or G418 selection.

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Visual Selection in Rice: A Strategy for the Efficient Identification of Transgenic Calli Accumulating Transgene Products

Hiroaki Saika, Haruko Onodera, and Seiichi Toki

Abstract

Fluorescent proteins such as green fluorescent protein (GFP) allow direct visualization of transformed cells without the need for exogenous substrates. Furthermore, visual selection using GFP is a powerful tool that can be used to isolate transformed cells without antibiotic or herbicide pressure and can be applied to transformation systems in plants hypersensitive to these agents. Moreover, we propose that visual selection enables isolation of calli in which the gene of interest is expressed to a high level, by selecting calli in which a strong GFP signal is observed. However, until now, the efficiency of clonal propagation using visual selection has been lower than that in antibiotic selection because of the technical difficulties involved in the isolation and clonal propagation of transformed calli with conventional transformation frequencies. We have succeeded in improving the efficiency of clonal propagation by the use of a rice cultivar that exhibits high competency for *Agrobacterium*-mediated transformation.

Key words: *Agrobacterium*-mediated transformation, Green fluorescent protein, Rice (*Oryza sativa* L.), Transformation frequency, Visual selection

1. Introduction

An appropriate selection marker is necessary for the successful selection of transformed cells from a large number of nontransformed cells. In order to produce widely acceptable transgenic crops, it is considered important to excise marker genes or to employ markers using selective agents that are less toxic to humans and the environment than antibiotics (1). Fluorescent proteins such as green fluorescent protein (GFP) enable real-time observation as well as easier and more precise evaluation of transformation

events under living conditions, without the need for treatment with exogenous substrate. Visual selection using GFP allows transformed cells to be isolated without any antibiotic or herbicide pressure, and is thus thought to be a useful technology for low-risk genetic engineering for crop improvement. This transformation technology can also be applied to plants that show a hypersensitive response to antibiotics or herbicides. Moreover, the intensity of GFP fluorescence was shown to be an accurate tool for protein quantification and can be used as a quantitative selection marker and indicator of gene expression (2–4). Calli in which the gene of interest (GOI) is expressed to a high level can be isolated by the visual selection of callus emitting strong GFP fluorescence (5).

2. Materials

2.1. *Agrobacterium*-Mediated Transformation Using Primary Callus

2.1.1. Plant and *Agrobacterium* Materials

1. Mature rice seeds (*Oryza sativa* L., 100–200 seeds, stored at 4°C). It is desirable to use rice seeds within 1 year of harvest because of the lower efficiency of callus formation and transformation in older seeds. We have been successful in applying visual selection using cvs. Nipponbare and Kasalath, which are model *japonica* and *indica* cultivars, respectively (5, 6).
2. *Agrobacterium tumefaciens* strain EHA105 (7). EHA105 is reported to be more suitable than LBA4404 for *Agrobacterium*-mediated transformation using callus derived from mature seeds of Nipponbare and Kasalath (8).
3. Binary vectors (see Note 1).

2.1.2. Media for *Agrobacterium*-Mediated Transformation (9)

All media are stored at 4°C. Each dish (9 cm in diameter, 2 cm in depth) receives 50 mL autoclaved medium. Plates are stored at room temperature; the lids are opened on a sterile bench just before use to allow any water drops on the surface of the medium to dry.

1. N6D medium: 30 g/L sucrose, 0.3 g/L casamino acids, 2.878 g/L L-proline, 2 mg/L glycine, N6-vitamins (100 mg/L myoinositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, and 1 mg/L thiamine HCl), N6 salts [Chu(N6)Medium Salt Mixture, Wako Pure Chemical Industries, Osaka, Japan], 2 mg/L 2,4-Dichlorophenoxy acetic acid (2,4-D), and 4 g/L gelrite (Wako Pure Chemical Industries), pH 5.8. Autoclave.
2. AB medium: 5 g/L glucose, 3 g/L K_2HPO_4 , 1.3 g/L $NaH_2PO_4 \cdot 2H_2O$, 1 g/L NH_4Cl , 150 mg/L KCl, 10 mg/L $CaCl_2 \cdot 2H_2O$, 2.5 mg/L $FeSO_4 \cdot 7H_2O$, and 15 g/L Bacto agar, pH 7.2. After autoclaving, add 1.2 mL of 1 M $MgSO_4 \cdot 7H_2O$ and the appropriate selective agent depending on the vector and *Agrobacterium* strain.

3. AAM medium (liquid medium): 68.5 g/L sucrose, 36 g/L glucose, 250 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 150 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 150 mg/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 3 g/L KCl, 40 mg/L Fe-EDTA, 10 mg/L $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.75 mg/L KI, 3 mg/L H_3BO_3 , 0.25 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 100 mg/L myoinositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 0.5 g/L casamino acids, 7.5 mg/L glycine, 176.7 mg/L L-arginine, 0.9 g/L L-glutamine, and 0.3 g/L L-aspartic acid, pH 5.2. Autoclave.
4. 2N6-AS medium: 30 g/L sucrose, 10 g/L glucose, 0.3 g/L casamino acids, 2 mg/L glycine, N6-vitamins, N6 salts, 2 mg/L 2,4-D, and 4 g/L gelrite, pH 5.2. After autoclaving, add 0.2 mL of 100 mg/mL acetosyringone.

2.1.3. Stock Solutions

1. 2,4-D: 5 mg/mL. Dissolve the powder in dimethyl sulfoxide (DMSO). Store at 4°C.
2. Acetosyringone (3',5',dimethoxy-4'-hydroxy-acetophenone): 100 mg/mL. Dissolve the powder in DMSO. Store at 4°C.
3. Meropenem (Wako Pure Chemical Industries): 12.5 mg/mL. Dissolve the powder in hot ddH₂O. Store at 4°C (see Note 2).

2.2. Observation of GFP Signals and Visual Selection

1. A fluorescent stereo microscope with a GFP2 filter (MZ FLIII, Leica Microsystems, Wetzlar, Germany, see Note 3).
2. Selection medium: After autoclaving N6D medium (see Sub-headings 2.1.2 item 4), add 1 mL of 12.5 mg/mL meropenem.

3. Methods

Agrobacterium-mediated transformation technology has been established in various cultivars of rice and is used widely in the fields of both molecular breeding and functional genomics. To date, visual selection using a GFP marker without antibiotic selection in rice has been reported in biolistic-mediated transformation of immature embryos and *Agrobacterium*-mediated transformation of callus derived from mature seeds (5, 10). In our experiment using Nipponbare, the efficiency of clonal propagation (the ratio of the number of lines successfully clonally propagated to that of *Agrobacterium*-infected calli) obtained by visual selection was estimated at 10–20%, compared to almost 90% achieved using antibiotic selection. This low efficiency is due to the technical difficulty of clonal propagation of transformed calli selected on the basis of GFP fluorescence (5). The difficulty of maintaining preferential growth of transformed cells using visual selection has been

pointed out in rice, sugarcane, and wheat following transformation by bombardment (10–12). A highly efficient transformation system is an absolute requirement for efficient visual selection, as transformed cells must be separated completely from the much larger fraction of untransformed cells (13). We showed that Kasalath shows higher competence for *Agrobacterium*-mediated transformation than Nipponbare, which enhanced the frequency of clonal propagation by three to eightfold compared to Nipponbare (6, 14). Thus, we demonstrated that a high-transformation frequency can improve the efficiency of clonal propagation by visual selection. Here, we illustrate our efficient visual selection protocol based on this highly efficient transformation system.

3.1. *Agrobacterium*-Mediated Transformation Using Primary Callus

1. The glumes are removed from mature seeds with a husker. The condition of dehulled seeds must be checked. For example, any abnormally coloured seeds, such as those with a green and brown patchy pattern, or seeds wounded by worms, should not be used because of the high risk of bacterial contamination during culture. Good quality dehulled seeds are placed in a 50-mL tube. Seeds are washed with 70% (v/v) ethanol for 10–30 s and subsequently rinsed with sterilized water several times. The seeds are then washed with 40 mL of 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween 20 vigorously for 15 min. After discarding the solution, the seeds are washed with sterilized water several times until bubbles derived from Tween 20 disappear. The seeds are then washed again with 40 mL of 2.5% (v/v) sodium hypochlorite solution vigorously for 15 min. After discarding the solution, the seeds are washed several times with sterile water, then placed on sterilized filter paper to remove excess water (see Note 4). Seeds are then placed on N6D medium and incubated at 33–36°C for 5–7 days (see Note 5).
2. *Agrobacterium* from a glycerol stock is inoculated onto AB medium containing appropriate antibiotics in Petri dishes and incubated at 28°C in the dark for 3–4 days before *Agrobacterium* cocultivation.
3. Primary calli are separated from seeds and seedlings using two forceps and collected in a 50-mL tube. Proliferating *Agrobacterium* are taken from AB medium with disposable tips or a spatula and suspended thoroughly in 40 mL of AAM medium supplemented with 20 mg/L acetosyringone in a fresh 50-mL tube. We usually use *Agrobacterium* suspension adjusted to $OD_{600}=0.1-0.01$ (see Note 6). This bacterial suspension is then poured into the 50-mL tube containing calli. The tube is shaken gently for 1.5 min. After discarding the bacterial suspension, calli are placed separately (not touching) on 2N6-AS

medium, onto which a sterilized paper filter has been placed and moistened with 0.5 mL of AAM medium supplemented with 20 mg/L acetosyringone. Calli on 2N6-AS medium are cocultivated at 25°C in the dark for 3 days. Calli should not be incubated at temperatures above 28°C in this step.

4. Cocultivated calli are collected in a 50-mL tube. Calli are washed with sterilized water >7–8 times and subsequently washed with 12.5 mL/L meropenem solution more than twice to remove *Agrobacterium* growing on the calli. Washed calli are placed onto sterilized filter paper to remove excess solution. Nine pieces of calli are placed onto the selection medium and grown at 33–36°C.

3.2. Observation of GFP Signals and Visual Selection

1. GFP signals are observed under a fluorescent stereo microscope with a GFP filter 10–14 days after *Agrobacterium* elimination. Cell clusters emitting green fluorescence are transferred to fresh selection medium and grown at 33–36°C (see Note 7).
2. This step is repeated 3–6 times until clonal cells emitting green fluorescence are successfully propagated (see Note 7). Clonally propagated calli can be cultured in liquid medium or transferred to regeneration medium (see Note 8).

4. Notes

1. For the efficient isolation of transformed calli hyperaccumulating a recombinant protein, a GOI–GFP fused gene expression cassette is required (see Fig. 1). We used sGFP(S65T) as a visible marker in visual selection (4). We discovered that a few rice calli transformed with GFP or DsRed could be visualized as light green or red under white light (data not shown, ref. 15). Thus, for easy discrimination between transformed and nontransformed calli under white light, it is important to select an appropriate visible marker. We felt that red fluorescence may be better than green fluorescence for observation under white light.
2. Application of meropenem and moxalactam—novel β -lactam antibiotics—was reported to improve transformation efficiency compared to carbenicillin and cefotaxime in rice (16).
3. It is important to reduce the background fluorescence emitted by rice callus and to ensure that the expressed fluorescence is strong. In our experiments, observation with a GFP2 filter (Ex. 480/40 nm and Em. 510 LP) was found to be better than with GFP3 (Ex. 470/40 nm and Em. 525/50 nm).

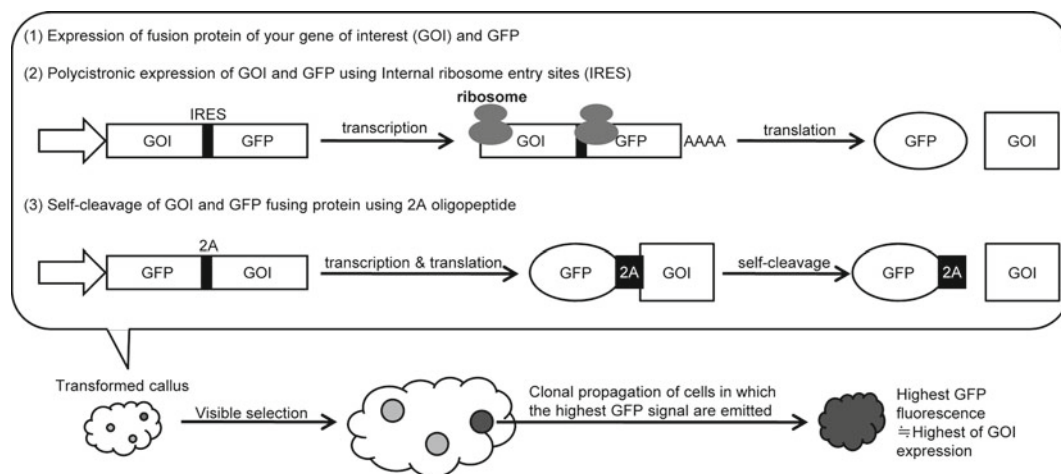


Fig. 1. Schematic representation of efficient isolation of transformed calli hyperaccumulating recombinant protein using visual selection. It has proved difficult to isolate calli hyperaccumulating recombinant proteins using visual selection using vectors in which GFP and GOI expression cassettes are present in tandem. This is reported to be because there is no correlation between the expression levels of genes aligned in tandem on T-DNA. We propose that the GOI should be fused to the *gfp* gene to ensure a positive correlation of the expression of both genes. In addition to a conventional GFP–GOI fusion gene (1), to achieve separate expression of the target protein, the inclusion of an internal ribosome entry sites (IRES) to allow cap-independent translation initiation (2), or 2A oligopeptides to mediate self-cleavage at the C-terminus that could allow efficient separation of GFP and the recombinant protein (3), is the strategy that could be considered (18–20). Both these latter systems have recently been reported to appropriate for the coordination of the expression of two genes, with the 2A oligopeptides system showing greater efficiency in rice endosperm (21). In the selection step, calli emitting the strongest GFP fluorescence are propagated; the positive association between estimated GFP protein concentration and GFP fluorescence (2) assumes that the GFP-target fusion gene is expressed to a high level in such calli.

4. When using rice cultivars such as Kasalath that have coloured (brown) pericarp, you may find that the colour in the pericarp is almost destained by sodium hypochlorite in this step.
5. You should bury rice seeds deeply so that the scutellum is in touch with the surface of the N6D medium in order to promote the growth of healthy callus; however, complete burial of seeds in the N6D medium is not recommended. We confirmed that rice primary callus derived from mature seeds grows vigorously at 37°C.
6. A higher *Agrobacterium* density results in more transformation events in primary callus (14). However, overgrowth of *Agrobacterium* in the cocultivation step and contamination by *Agrobacterium* in the selection step are thought to cause a decrease in transformation efficiency. To prevent this, you should use appropriate culture conditions such as high-temperature conditions (9) and efficient antibiotics (see Note 2).
7. In the first and second transfer, since only a few cells emitting green fluorescence may be observed, especially in Nipponbare, cell clusters containing cells emitting green fluorescence should be transferred to fresh medium. On fresh N6D medium, cell clusters

are dissected with forceps. Direct observation and manipulation under a fluorescent stereo microscope on a sterile bench is the best method of isolation of green fluorescence emitting cells. However, we have performed visual selection successfully using a fluorescent stereo microscope without a sterile bench as follows: the location where green fluorescence-emitting callus is observed is marked on the surface of the lid of the dish. On a clean bench, the lid of dish is placed under the dish. Thus, you can isolate the calli required by following the marks seen on the lid of the dish.

8. To obtain regenerated plants, the duration of callus culture should be as short as possible. Long periods of cell culture in a dedifferentiated state results in Tos17 transposition (17) and callus browning derived from polyphenol accumulation and cell death, which prevents further proliferation, especially in callus of *indica* rice.

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Chapter 8

Characterization of Rice Genes Using a Heterologous Full-Length cDNA Expression System

Mieko Higuchi, Youichi Kondou, Masaki Mori, Takanari Ichikawa, and Minami Matsui

Abstract

As a result of the progress in sequencing technology, many plant genomes have now been determined. Functional genomics is required to clarify gene function in many of these species. To identify useful genes easily and quickly, we have developed a FOX (full-length cDNA overexpressor) hunting system in which full-length cDNAs are overexpressed in *Arabidopsis* plants. This system was applied to high-throughput analysis of rice genes through heterologous expression in *Arabidopsis* (rice FOX *Arabidopsis* lines). We demonstrated that it is possible to carry out high-throughput analysis of gene function by utilizing rice FOX *Arabidopsis* lines. In this protocol, we describe how to isolate candidate rice FOX *Arabidopsis* lines and to determine the rice fl-cDNA that is responsible for the observed phenotype.

Key words: Full-length cDNA, *Arabidopsis*, FOX hunting system, Heterologous gene expression, Transgenic plants

1. Introduction

The use of *Arabidopsis* as a model system in plant research has been a powerful tool to investigate gene function, and we can apply the information obtained from *Arabidopsis* research to other plant species. Since the completion of the *Arabidopsis* genome sequence and as a result of the progress in sequencing technology, the genomes of many other plant species, including rice, poplar, grape, papaya, and sorghum, have been revealed (1–5). Subsequently, in the post-genomic era, researchers have focused on understanding the biological function of every gene product. One of the most important tools for dissecting gene function is phenotypic screening because a phenotype associated with a gene function is often the best clue

to the gene's role. In *Arabidopsis* and rice, large collections of gene-indexed mutants have been created to help uncover the biological function of particular genes (6). However, these genomic approaches are not yet practical in many crop species for which the transformation method is inefficient or not available or the plant size is not suitable for a collection of mutant resources.

One approach, heterologous expression of genes, may be efficient as a genome-wide approach in crops. Using this strategy, we can gain an understanding of gene function in different plant species. We generated gain-of-function mutant lines caused by ectopic expression of full-length cDNAs (fl-cDNAs) in *Arabidopsis* and named the approach the FOX hunting system (full-length cDNA overexpressor gene hunting system) (7). This system can be used for heterologous expression of genes because only fl-cDNAs are required for the functional analysis of genes. We applied the FOX hunting system to the characterization of rice genes as a model heterologous expression system (8). A rice fl-cDNA expression library was generated by expressing around 13,000 normalized rice fl-cDNAs under the control of the *CaMV* 35S promoter. This expression library was introduced into *Agrobacterium* to generate an *Agrobacterium* expression library. This *Agrobacterium* library was used to transform *Arabidopsis* plants by *in planta* transformation. In this way, we generated more than 33,000 independent *Arabidopsis* transgenic lines that expressed rice fl-cDNAs (rice FOX *Arabidopsis* lines). These lines are distributed from the Plant Science Center (<http://www.psc.riken.jp/english/index.html>).

High-throughput screens using these mutant populations should provide a way to analyze plant gene function. We screened the rice FOX *Arabidopsis* lines to isolate putative mutants with alterations in a range of phenotypes, including morphology, photosynthesis, element and pigment accumulation, hormone profiles, secondary metabolites, pathogen resistance, salt tolerance, UV signaling, high light tolerance, and heat-stress tolerance. Two heat-tolerant rice FOX *Arabidopsis* lines, one expressing *OsHsfA2e* that encodes a heat-stress transcription factor (9) and the other expressing *ONAC063* that encodes a NAC transcription factor (10), were isolated from the rice FOX *Arabidopsis* lines. We isolated a rice FOX *Arabidopsis* line expressing rice ferredoxin NADP⁺ reductase (FNR) 1, and overexpression of *FNRI* in rice plants led to the same phenotype as seen in the original rice FOX *Arabidopsis* line (11). One novel disease resistance gene designated *BROAD-SPECTRUM RESISTANCE1* (*BSRI*) that confers resistance to both bacterial and fungal pathogens in *Arabidopsis* and rice was isolated by this system (12). These findings suggested that rice gene function could be analyzed by employing *Arabidopsis* as a heterologous host. In this protocol, we will describe how to generate rice FOX *Arabidopsis* lines using an *Agrobacterium* library, identify rice fl-cDNAs using the FOX hunting system, and investigate their function in rice.

1.1. Outline

The overall process is shown in Fig. 1. An *Agrobacterium* library harboring rice fl-cDNAs was used to generate rice FOX *Arabidopsis* lines. T₁ *Arabidopsis* plants expressing rice fl-cDNAs are self-pollinated, and the T₂ seeds can be used for screening. T₁ seeds can also be used, but antibiotic selection must be applied in parallel. The introduced fl-cDNA of interest is isolated from the candidate FOX line and then reintroduced into *Arabidopsis* to confirm that its expression is responsible for the observed phenotype. Finally, it is transformed into rice under the control of the maize ubiquitin promoter (13) to determine whether the mutant phenotype observed in *Arabidopsis* is recapitulated in rice.

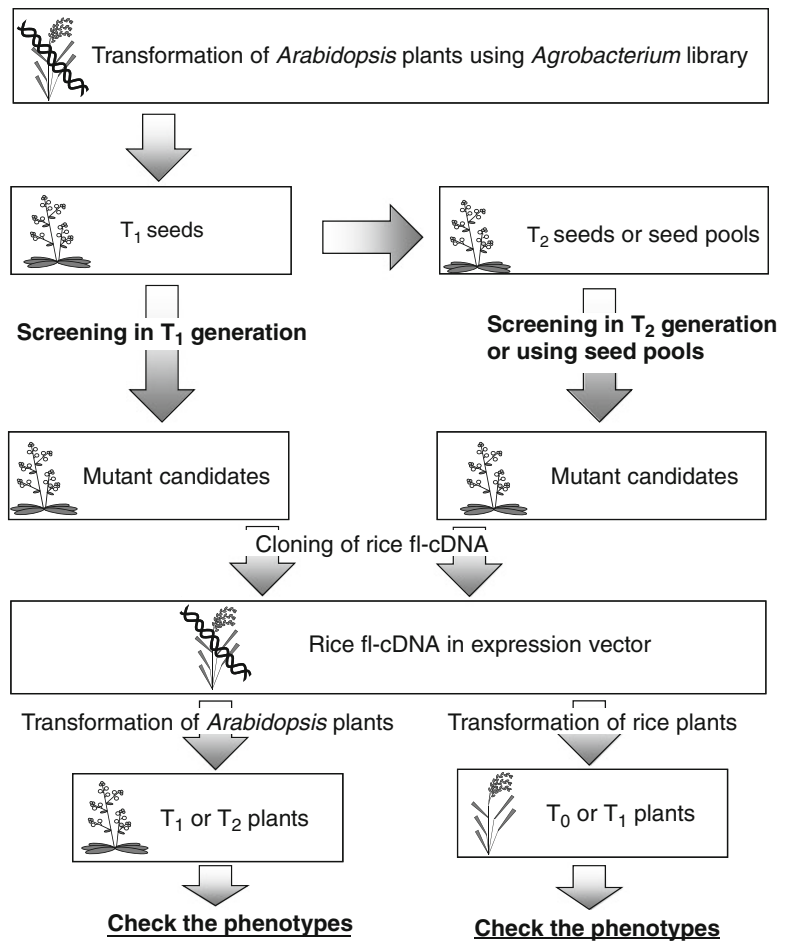


Fig. 1. Flow diagram for isolation and determination of rice fl-cDNAs causing various phenotypes. The *Agrobacterium* library contains a rice fl-cDNA library harbored in an expression vector suitable for *Arabidopsis*. T₁ seeds, T₂ seeds, and seed pools of rice FOX *Arabidopsis* lines can be used for screening under various conditions. Screening in the T₂ generation may be replaced by a search in the rice FOX database. The *Agrobacterium* library and T₂ seeds of independent rice FOX lines are available from the RIKEN Plant Science Center.

2. Materials

2.1. Culture of *Escherichia coli* and *Agrobacterium* Cells

1. The strains used are DH10B for *Escherichia coli* and GV3101pMP90 and EHA105 for *Agrobacterium*. The *Agrobacterium* library and T₂ seeds of independent rice FOX lines are available from the RIKEN Plant Science Center. *E. coli* competent cells are purchased from Invitrogen (ElectroMAX DH10B, Invitrogen Inc.), and SOC medium is supplied with competent cells (see Note 1).
2. For LB medium, 10 g of tryptone peptone, 5 g of yeast extract, and 5 g of NaCl are dissolved in 1 L of water, and the medium is autoclaved. Before autoclaving, 16 g of agarose is added to make LB agar medium. Antibiotics are added after autoclaving.
3. For the infiltration medium, 2.3 g of Murashige and Skoog (MS) inorganic salts (Nihon Pharmaceutical Co., Ltd.), 50 g of sucrose, 112 μ L of Gamborg's vitamin solution (Sigma-Aldrich Co.), and 200 μ L of Silwet L-77 (Agri-Turf Supplies Inc.) are mixed in 1 L of water.
4. To prepare stock solutions, 100 mg of kanamycin, 20 mg of gentamicin, and 50 mg of hygromycin B are dissolved in 1 mL of water. Sterilization is achieved by filtration through a 0.22- μ m membrane. The stock solutions should be stored at -20°C .

2.2. Transformation of *Arabidopsis* Plants

1. pBIGS2113SF is used as the expression vector for transformation of *Arabidopsis*.
2. Ecotype Columbia-0 is used as the *Arabidopsis thaliana* WT plant. They are grown at 22°C under long-day conditions (16 h light and 8 h dark) (see Note 2).
3. 1.5 kg of PRO-MIX (Premier Tech Ltd) and 0.9 kg of vermiculite (Fukushima VERMI KK) are mixed with an appropriate volume of water containing a 1,000-fold dilution of HYPONeX (HYPONeX Japan Corp., Ltd.) and used to cultivate the *Arabidopsis* plants. The soil mix is autoclaved at 120°C for 30 min before use.
4. For basic agar medium (BAM), 101 mg of KNO_3 and 8 g of Bacto Agar are dissolved in 1 L of water, and the medium is autoclaved (14). Antibiotics are added after autoclaving (see Note 3).
5. For 0.2% agar, 2 g of Bacto Agar is dissolved in 1 L of water, and the agar solution is autoclaved.
6. To prepare a stock solution, 100 mg of cefotaxime sodium salt is dissolved in 1 mL of water. Sterilization is achieved by filtration through a 0.22- μ m membrane. The stock solution should be stored at -20°C .

2.3. Cloning of Isolated Fl-cDNA into pBIGS2113SF and pRiceFOX

1. For PCR amplification, 25 μL of 2 $\times$ PrimeSTAR GC buffer I, 4 μL of the dNTP mixture, 1 μL of each 10- μM primer, an appropriate volume of DNA template, and 1.25 U of PrimeSTAR HS DNA polymerase (Takara Bio Inc.) are mixed with distilled water to give a final volume of 50 μL (see Note 4). The 2 $\times$ PrimeSTAR GC buffer I and dNTP mixture are supplied with the polymerase.
2. *Sfi*I is purchased from Takara Bio Inc. 10 $\times$ H buffer and the BSA solution are supplied with the restriction enzyme.
3. T4 DNA ligase (400 U/ μL) is purchased from New England Bio Labs Inc. 10 $\times$ Ligation buffer is supplied with the ligase.
4. For 3 M sodium acetate, 40.8 g of NaOAc $\cdot$ 3H₂O is dissolved in 100 mL of water, and the pH of the solution is adjusted to 4.8 with glacial acetic acid. The solution is then autoclaved.

2.4. Transformation of Rice Plants

1. pRiceFOX (13) is used as the expression vector for transformation of rice.
2. *Oryza sativa* ssp. *japonica* cv. Nipponbare is used as the WT of rice. Mature seeds harvested from rice plants grown in paddy fields are used for transformation.
3. To make a stock solution, 200 mg of carbenicillin is dissolved in 1 mL of water. Sterilization is achieved by filtration through a 0.22- μm membrane. The stock solution should be stored at -20°C .
4. To prepare a stock solution, 100 mg of acetosyringone is dissolved in 1 mL of DMSO and stored at -20°C .
5. N6 vitamins ($\times$ 1,000): 200 mg of glycine, 50 mg of nicotinic acid, 50 mg of pyridoxine HCl, 100 mg of thiamine HCl, and 10 g of myoinositol are dissolved in 100 mL of water and stored at -20°C in 1 mL aliquots.
6. MS vitamins ($\times$ 1,000): 200 mg of glycine, 50 mg of nicotinic acid, 50 mg of pyridoxine HCl, 10 mg of thiamine HCl, and 10 g of myoinositol are dissolved in 100 mL of water and stored at -20°C in 1 mL aliquots.
7. All media are poured in sterile Petri dishes (9-cm diameter) except for the liquid AAM medium.
8. N6D medium, pH 5.8: N6 salts (Nihon Pharmaceutical Co., Ltd.) and N6 vitamins with 30 g/L sucrose, 0.3 g/L casamino acid, 2.9 g/L proline, and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) solidified with 4 g/L Gelrite.
9. AB medium, pH 7.2: 5 g/L glucose, 3 g/L K₂HPO₄, 1.3 g/L NaH₂PO₄ $\cdot$ 2H₂O, 1 g/L NH₄Cl, 150 mg/L KCl, 10 mg/L CaCl₂ $\cdot$ 2H₂O, 2.5 mg/L FeSO₄ $\cdot$ 7H₂O, and 296 mg/L MgSO₄ $\cdot$ 7H₂O solidified with 15 g/L Bacto Agar.

10. AAM medium (15) has the addition of 40 mg/L acetosyringone. Acetosyringone is added after autoclaving.
11. 2N6-AS medium, pH 5.2: N6 salts and N6 vitamins with 30 g/L sucrose, 10 g/L glucose, 0.3 g/L casamino acid, 2 mg/L 2,4-D, and 20 mg/L acetosyringone solidified with 4 g/L Gelrite. Acetosyringone is added after autoclaving.
12. RE-III medium, pH 5.8: MS salts and MS vitamins with 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acid, 0.02 mg/L naphthaleneacetic acid (NAA), 2.0 mg/L kinetin, 200 mg/L carbenicillin, and 50 mg/L hygromycin B solidified with 4 g/L Gelrite. Carbenicillin and hygromycin B are added after autoclaving.
13. Hormone-free medium, pH 5.8: MS salts and MS vitamins with 30 g/L sucrose and 50 mg/L hygromycin B solidified with 4 g/L Gelrite. Hygromycin B is added after autoclaving.
14. 1/2× MS medium, pH 5.8: half-strength MS salts with 30 g/L sucrose and 50 mg/L hygromycin B solidified with 4 g/L Gelrite. Hygromycin B is added after autoclaving.

3. Methods

3.1. Construction of Rice FOX Arabidopsis Lines Using Agrobacterium Library

3.1.1. Transformation of Rice Fl-cDNAs in pBIGS2113SF into Arabidopsis Plants Through Agrobacterium

1. The culture containing the *Agrobacterium* library is thawed completely on ice. The *Agrobacterium* cells harbor pBIG-S2113SF into which the rice fl-cDNA library is integrated.
2. Add 2–4 µL of the *Agrobacterium* library to 200 mL of LB medium with 100 µL of both the kanamycin and the gentamicin stock solutions (final concentrations 50 and 10 µg/mL, respectively).
3. The *Agrobacterium* culture is incubated with vigorous shaking at 28°C until the OD₆₀₀ reaches 1.2–1.5.
4. The culture is transferred into a centrifuge tube and spun at 6,000×g for 15 min to harvest the cells.
5. The supernatant is removed by decantation. The pellet of *Agrobacterium* culture is resuspended in approximately 200 mL of infiltration medium. The OD₆₀₀ should be about 0.8.
6. The pots in which the *Arabidopsis* plants are growing are inverted, and the inflorescences are completely dipped into the *Agrobacterium* suspension for 30 s.
7. The pots are placed inside plastic bags. The bags are sealed, and the plants returned overnight to normal growing conditions.
8. The next day, the bags are opened, and the plants are left overnight again.

9. The pots are removed from the plastic bags and put under normal conditions to grow on for harvesting of the T_1 seeds.

3.1.2. Isolation of Transgenic *Arabidopsis* Plants

1. Put 0.25 g of T_1 seeds into a 15-mL conical tube and add 10 mL of 70% ethanol (see Note 5).
2. After shaking of the tube, the 70% ethanol is removed, and then, 10 mL of 10% sodium hypochlorite containing 0.1% Triton X-100 is added.
3. The solution is decanted after 10 min, and the seeds are washed with sterile water three times.
4. The water is removed after washing, and 5 mL of 0.2% agar is added to the tube.
5. After inversion of the tube, the seeds in agar are spread on Petri dishes (100 mm × 140 mm) containing BAM agar medium with 20 µg/mL of hygromycin B for selection of transformed seedlings and 100 µg/mL of the cefotaxime sodium salt.
6. Petri dishes are sealed with surgical tape and put at 4°C and left for at least 2 days in the dark to induce germination.
7. The Petri dishes are transferred to normal growth conditions for 10–14 days.
8. Seedlings that have true leaves and extended main roots are picked from the Petri dishes and transferred to soil. It may take longer to identify and pick all transformants because some transformed seedlings show growth retardation phenotypes.
9. The seedlings are grown up until the T_2 seeds are harvested.

3.2. Screening of Rice FOX *Arabidopsis* Lines by Various Indexes

3.2.1. Screening in T_1 Generation

1. If screening is carried out in the T_1 generation of the rice FOX *Arabidopsis* lines, the plants can be grown under varying conditions in parallel with antibiotic selection. For example, when T_1 plants grown on plates are subjected to environmental stresses, such as high temperature and high light, resistant plants can be picked and transferred to soil.
2. It is better to check the phenotypes in the T_2 generation after the harvest of the T_2 seeds.

3.2.2. Screening in T_2 Generation

1. If screening is performed in the T_2 generation, select the rice FOX *Arabidopsis* lines under screening conditions (see Note 6).
2. Alternatively, it can be done using the rice FOX database (<http://ricefox.psc.riken.jp/>). Many rice FOX *Arabidopsis* lines showing various phenotypes in the T_1 and/or T_2 generation are registered in the database (16). If candidates showing interesting phenotypes are identified, T_2 seeds can be ordered from the RIKEN Plant Science Center.

3.2.3. Screening Using Seed Pools

1. Seed pools, which have been ordered from the RIKEN BioResource Center (<http://www.brc.riken.jp/lab/cpd/Eng/>), can be used for screening rice FOX *Arabidopsis* lines. Seed pools can also be made by the mixing of independent T₂ seeds.
2. Selected plants are grown until the harvesting of seeds of the next generation.
3. After harvesting, the phenotypes are checked under screening conditions.

3.3. Determination of Relationship Between Rice Fl-cDNA and Heterologous Phenotypes in *Arabidopsis*

3.3.1. Isolation of Rice Full-Length cDNA from Rice FOX *Arabidopsis* Lines

1. Chromosomal DNA is isolated from the rice FOX *Arabidopsis* lines that are selected as putative mutants.
2. The PCR solution is made according to the instructions of the DNA polymerase. More than 200 ng of chromosomal DNA is added to a 50-μL reaction. The F1 and R1 primer set in Table 1 is recommended for amplification of rice fl-cDNA in rice FOX *Arabidopsis* plants.
3. After denaturation at 94°C, 30 cycles of PCR are carried out: 94°C for 30 s, 52°C for 30 s, and 72°C for 3 min. At the end of the PCR, mixture is incubated at 72°C for 10 min for a final extension.
4. The PCR products are electrophoresed in a 0.8% agarose gel, and the bands are cut out for isolation of the amplified PCR fragments (see Notes 7 and 8). The PCR fragments derived from rice cDNAs may be amplified using other primer sets in Table 1 if PCR with the F1 and R1 primer set is unsuccessful. Alternatively, nested PCR using the first PCR products and an internal primer set from Table 1 can be performed.
5. After purification from the agarose gel, the PCR fragments are sequenced to determine the transformed rice fl-cDNAs (see Note 9).

3.3.2. Cloning of Isolated Fl-cDNA into pBIGS2113SF

1. Add 0.9 μL of pBIGS2113SF, 1 μL of 10× H buffer, 0.1 μL of the BSA solution, and 0.5 μL of *Sfi*I to 7 μL of the purified PCR fragments harboring the rice fl-cDNA.
2. After incubation at 50°C overnight, 0.5 μL of *Sfi*I is added to the reaction solution, and the tubes are incubated at 50°C for 3 h. The tubes are spun down every hour during the 3-h incubation to bring down condensation from the lids of the tubes.
3. Add 1 μL of 3 M sodium acetate and 10 μL of isopropanol to the reaction tubes, and put at -20°C for at least 30 min to precipitate the DNA.
4. After centrifugation of the tubes at 4°C for 20 min, the DNA pellets are washed twice with 70% ethanol and dried.

Table 1
Primer sets used for amplification and sequencing of rice fl-cDNAs using FOX lines

Forward primer		Reverse primer	
Name	Sequence	Name	Sequence
F1	GGAAGTTCATTATTTCGGAGAG	R1	GGCAACACAGGATTCAATCTTAAG
F2	CATTATTTCGGAGAGGTACGTAT	R2	GGATTCAATCTTAAAGAAACTTTTATTGCCAA
F3	GTACGTATTTTACAAACAATTACCAACAAC	R3	CAAATGTTTGAACGATCGGGGAAAT
F4	ATTACATTTTACATTCTACAACTACATCT	R4	GATCCTCTAGAGGCCCTTAT

The smaller number of the primer name indicates more distant location from the *Sfi*I site in pBIG2113SF

5. To resuspend the DNA, 8.5 μL of distilled water and 1 μL of 10 $\times$ ligation buffer are added to the tubes, and then, 0.5 μL of T4 DNA ligase is added.
6. The tubes are incubated at 16°C overnight, and then, 1 μL of 3 M sodium acetate and 25 μL of ethanol are added to the reaction tubes.
7. The tubes are incubated at -20°C for at least 30 min and then centrifuged at 4°C for 20 min. The DNA pellets are washed with 70% ethanol and resuspended in 5 μL of distilled water (see Note 10).
8. In preparation for electroporation, 45 μL of competent cells of the *E. coli* strain DH10B is added to the tubes. After mixing, the cells are transferred into an ice-cold cuvette.
9. The cells are electroporated at 1.5 kV 200 Ω , and 25 μF for 4 ms. Immediately afterward, 500 μL of SOC medium is added and mixed by pipetting.
10. The cells are transferred into tubes and incubated at 37°C for 1 h.
11. All the cells are spread on Petri dishes containing LB agar medium with 50 $\mu\text{g}/\text{mL}$ of kanamycin and incubated at 37°C overnight.
12. Cells are picked from kanamycin-resistant colonies and added to PCR tubes containing colony PCR mix. If the PCR fragment cannot be cloned into the expression vector, increase the amount of the PCR fragment and expression vector in the reaction mixture during *Sfi*I digestion.
13. PCR is carried out under the same conditions as for the amplification of the rice fl-cDNA. The PCR products are electrophoresed in a 0.8% agarose gel for confirmation of the size of the PCR fragments. Continue to the next step if the amplified PCR fragments are of the expected size.
14. Cells are repicked from the colonies and put into 2 mL of LB medium with 1 μL of the kanamycin stock solution (final concentration 50 $\mu\text{g}/\text{mL}$).
15. After incubation at 37°C overnight, pBIGS2113SF harboring the rice fl-cDNA is isolated from the *E. coli* cells.
16. The rice fl-cDNA in pBIGS2113SF is sequenced to check for PCR errors.

3.3.3. Retransformation
of Isolated Fl-cDNA
in pBIGS2113SF into
Arabidopsis Plants
Through *Agrobacterium*

1. Add 1 μL of the plasmid DNA, pBIGS2113SF harboring the rice fl-cDNA, to 50 μL of competent cells of the *Agrobacterium* strain GV3101pMP90. After mixing, the cells are transferred to an ice-cold cuvette.
2. The cells in the cuvette are electroporated at 2.5 kV, 200 Ω , and 25 μF for 4 ms. Immediately afterward, 500 μL of SOC medium is added to the cuvette and mixed by pipetting.

3. The cells are transferred into tubes and incubated at 28°C for 1–3 h.
4. All the cells are spread on Petri dishes with LB agar medium containing 50 µg/mL of kanamycin and 10 µg/mL of gentamicin and are incubated at 28°C for 2–3 days.
5. The antibiotic-resistant cells are picked and added to 2 mL of LB medium with 1 µL of both the kanamycin and gentamicin stock solutions (final concentrations 50 and 10 µg/mL, respectively).
6. After incubation at 28°C overnight, 2 mL of the *Agrobacterium* culture is added to 200 mL of LB medium with 100 µL of both the kanamycin and gentamicin stock solutions (final concentrations 50 and 10 µg/mL, respectively).
7. *Arabidopsis* plants are retransformed using the *Agrobacterium* cultures as described in Subheadings 3.1.1 and 3.1.2.
8. The transformed *Arabidopsis* plants are grown to maturity for harvest of the T₁ seeds.
9. If the phenotype of the rice FOX *Arabidopsis* line is recaptured in the retransformed plants, analyze the expression level of the rice fl-cDNA in some independent retransformed lines. The strength of a phenotype will be correlated with the expression level of the introduced rice fl-cDNA.

3.4. Transformation of Isolated Fl-cDNA into Rice and Confirmation of Homologous Phenotypes

The rice transformation protocol described here is based on the high-speed transformation method described by Toki et al. (15).

3.4.1. Cloning of Isolated Fl-cDNA into pRiceFOX and Transformation of the Constructed Vector into *Agrobacterium* Cells

1. Isolated fl-cDNA is cloned into pRiceFOX as described in Subheading 3.3.2, except the vector used is pRiceFOX instead of pBIGS2113SF.
2. pRiceFOX is introduced into the *Agrobacterium* strain EHA105 by electroporation, as described in Subheading 3.3.3. Antibiotic selection is performed using 50 µg/mL of kanamycin and 30 µg/mL of hygromycin B at 28°C for 2 days.
3. The antibiotic-resistant cells are picked from colonies on LB agar medium and added to 2 mL of LB medium with 1 µL of the kanamycin (100 mg/mL) and 1.2 µL of the hygromycin B (50 mg/mL) stock solutions (final concentrations 50 and 30 µg/mL, respectively).
4. After incubation at 28°C overnight, 500 µL of the preculture is transferred to a sterile 1.5-mL tube, and 200 µL of sterile glycerol is added, mixed well, and stored at –80°C.

3.4.2. *Sterilization
and Sowing of Rice Seeds
for Callus Induction*

1. Approximately, 250 rice seeds are dehusked (see Note 11).
2. Dehusked seeds are transferred into a 50-mL conical tube. Add 40 mL of 70% ethanol, and mix by inverting the tube several times for 10–20 s.
3. After the 70% ethanol is removed, 40 mL of 2.5% sodium hypochlorite containing one drop of Tween 20 is added, and the tube is placed on a rotary shaker and shaken gently for 15 min.
4. The solution is decanted on a clean bench, and more of the same solution is added. The tube is shaken again for 15 min.
5. From this step onward, all manipulations should be carried out on a clean bench until transferring the transgenic plants to soil. After the solution is removed, the seeds are washed with sterile water by shaking the tube vigorously. This step is repeated ten times.
6. Seeds are poured on to sterile filter paper in a sterile Petri dish to remove the remaining water.
7. The sterilized seeds are transferred to the surface of N6D medium by sterile forceps; 25 seeds are placed neatly in one Petri dish.
8. Petri dishes are sealed with surgical tape and put in the growth chamber at 31°C under continuous light for 5 days.

3.4.3. *Preculture
and Infection with
Agrobacterium*

1. The *Agrobacterium* strain EHA105 harboring the isolated fl-cDNA in pRiceFOX is cultured on AB medium containing 50 mg/L of kanamycin and 50 mg/L of hygromycin B at 23°C in the dark for 3 days.
2. Add 40 mL of AAM medium to a 50-mL conical tube.
3. A sterile filter paper (9-cm diameter) is put on the 2N6-AS medium, and 0.5 mL of AAM medium is added and spread around.
4. One loopful of precultured *Agrobacterium* is scraped from the medium using a sterile microspatula and resuspended in the remaining AAM medium in the conical tube (see Note 12).
5. Rice seeds cultured for 5 days are collected in sterile Petri dishes using forceps, and the shoots and endosperm are removed. The remaining parts of the seeds containing calli on the scutellum are collected in a new 50-mL conical tube.
6. The *Agrobacterium* solution is added to the conical tube, and the tube is inverted gently for 90 s.
7. Calli on the scutellum are collected using a sterile tea strainer, and superfluous bacterial solution is removed by dabbing on sterile filter paper on a Petri dish.

8. They are transferred on to the filter paper on the 2N6-AS medium using forceps and positioned so there is no contact between them. Only one Petri dish is used.
9. The Petri dish is sealed with surgical tape and put in the growth chamber for cocultivation at 23°C in the dark for 3 days.

3.4.4. Washing and Selection of Calli

1. After 3 days, cocultivated calli are transferred to one conical tube and washed with 40 mL of sterile water five times to remove the *Agrobacterium*.
2. They are washed a further three times with sterile water containing 500 µg/mL of carbenicillin. Extra water is removed by putting them on sterile filter paper.
3. Individual callus pieces are transferred with sterile forceps on to N6D medium containing 400 µg/mL of carbenicillin and 50 µg/mL of hygromycin B. In one Petri dish, 25 calli are placed. Ten Petri dishes are used.
4. Petri dishes are sealed with surgical tape and put in the growth chamber at 31°C under continuous light for 10–14 days.
5. Actively growing calli are transferred to N6D medium containing 200 µg/mL of carbenicillin and 50 µg/mL of hygromycin B and again cultured for 10–14 days (see Note 13). In one Petri dish, 16 calli are placed. Ten Petri dishes are used.

3.4.5. Regeneration of Transgenic Plants

1. Actively growing calli with a creamy colour are transferred to regeneration medium (RE-III). Eight calli are placed in one Petri dish. Ten Petri dishes are used.
2. Petri dishes are sealed with surgical tape and put in the growth chamber at 31°C under continuous light for 10–14 days. During this stage, green spots appear on some calli.
3. Calli are transferred to fresh RE-III medium and cultured for 10–14 days again. Eight calli are placed in one Petri dish. Ten Petri dishes are used.
4. Regenerated shoots with roots are transferred to hormone-free medium and cultured for 10–20 days (see Note 14). Four regenerated plants are placed in one Petri dish.

3.4.6. Culture of Transgenic Plants

1. After 10–20 days, hygromycin-resistant shoots are potted into soil (see Note 15).
2. Regenerated plants (T_0 generation) are grown in an appropriate greenhouse (see Note 16).
3. After several months, T_1 seeds of the transgenic rice are harvested (see Note 17).
4. T_1 seeds are sown on $1/2\times$ MS medium with hygromycin B, and WT seeds are sown on the same medium without antibiotic,

and they are grown simultaneously in the growth chamber at 25–30°C under long-day conditions (16 h light and 8 h dark).

5. After 7–10 days, hygromycin-resistant T_1 shoots and WT shoots are transferred to soil. Phenotypes are investigated comparing the T_1 and WT plants (see Note 18).
6. If the expected phenotype is observed, the severity of the phenotype should be correlated with the expression level of the introduced rice fl-cDNA.

4. Notes

1. Please use highly competent *E. coli* cells. We use electrocompetent *E. coli* cells with a competency of at least 1×10^8 transformants/ μ g of pUC19 plasmid.
2. It is very important to use healthy *Arabidopsis* plants for good transformation efficiency. We usually use nine plants in one pot for floral dipping.
3. If hygromycin-resistant plants are not obtained, use 0.1 $\times$ MS plates for antibiotic selection. We have found that it is possible to pick more resistant plants from this medium.
4. We used PrimeSTAR HS DNA polymerase with GC buffer for amplification of rice fl-cDNAs. LA-Taq (Takara Bio Inc.) or KOD FX (Toyobo Co., Ltd.) is also recommended when it is difficult to amplify the fl-cDNAs.
5. When we sow 250 mg of T_1 seeds, we can pick about 30–40 transformants.
6. It is better to select candidate rice FOX *Arabidopsis* lines that show a dominant phenotype because the FOX hunting system is a gain-of-function technology.
7. A PCR fragment of approximately 200 bp is obtained when empty vector is used as the template DNA. Therefore, the PCR products larger than 200 bp should be recovered.
8. We have reported that on average, 2.6 *Arabidopsis* fl-cDNAs are inserted in the FOX *Arabidopsis* lines (7). As a result, multiple PCR fragments are detected when fl-cDNAs are amplified from the rice FOX *Arabidopsis* lines. All amplified fragments should be used to generate retransformed plants to confirm which fl-cDNA causes the phenotype.
9. To characterize the introduced rice fl-cDNA, direct sequencing of PCR fragments can be carried out using internal primers to those used for PCR amplification. It is better to perform sequencing from both ends. We found that different fl-cDNAs were tandemly inserted into the expression vector in some lines.

10. Ethanol precipitation of the ligated DNA is carried out to eliminate salts in the reaction mixture to encourage high transformation efficiency. Add 1 μ L of the ligation mixture to 30 μ L of *E. coli* competent cells when the ligation mixture is used directly in the transformation.
11. About 250 rice seeds are generally used for one *Agrobacterium* strain. Brown-coloured or thin seeds should not be used.
12. Scraping too much *Agrobacterium* reduces the transformation efficiency. The *Agrobacterium*-containing solution has to be fully resuspended by pipetting.
13. If growth of *Agrobacterium* is noted around the calli, the contaminated calli should not be transferred.
14. Sometimes, many shoots regenerate from one callus. If this occurs, only one individual shoot with roots is transferred from one callus.
15. Plants with brown shoots or roots should not be transferred. Generally, 20–50 transgenic plants are generated.
16. In many countries, growing transgenic plants is tightly regulated. For example, in Japan, transgenic plants can only be grown in a containment greenhouse. Long-day conditions (usually more than 13 h light) for more than 1 month are necessary for vegetative growth of Nipponbare rice.
17. Nipponbare rice requires short-day conditions (usually less than 11 h light) to induce reproductive growth after the period of long day required for vegetative growth.
18. It is not recommended to analyze phenotypes in T₀ plants in detail because residual hormones from tissue culture may affect the phenotype.

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Bioactive Bead-Mediated Transformation of Plants with Large DNA Fragments

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Abstract

The efficient transformation of plants with large DNA molecules containing a set of useful genes would provide vast possibilities for the genetic improvement of agricultural as well as nonagricultural plants. The development of the bioactive beads (BABs) transformation method has proven useful for introduction of large DNA molecules into plant cells. In this chapter, the BABs transformation method used for the transformation of a 100-kb BAC DNA construct containing wheat genes into rice will be presented. Furthermore, the improved production method for BABs will be described. With the conventional method for producing BABs, the bead size varies, and the larger beads tend to carry fewer DNA molecules than the smaller beads. Thus, in order to facilitate the preparation of BABs with more uniform sizes, a simple set-up composed of a sine wave sound generator and microsyringe pump was fabricated. Using this bead-maker set-up, uniform and smaller beads could be produced which enhance the transformation efficiency.

Key words: Bioactive beads, Plant transformation, Rice, Tobacco BY-2, Protoplast

1. Introduction

The use of bioactive beads (BABs) in plant transformation was first evaluated in tobacco BY-2 cells (1). BABs are small and spherical calcium alginate beads that can entrap DNA molecules and carry them into a recipient cell. Thus, BABs have been used for transformation and transfection to deliver different sizes of DNA molecules ranging from 4-kb simple plasmid DNA (1) to 468-kb YACs (2). Plant transformation using BABs has been shown to be feasible using protoplasts from tobacco (1, 3, 4), eggplant (3), carrot (3), and rice (5). Furthermore, regenerated plants have been obtained from tobacco (3) and rice (6) protoplasts that were transformed using BABs. The successful introduction of the *Aegilops tauschii* genomic region with about 100 kb in size, containing grain hardness

genes into rice was carried out using BABs transformation and resulted in homozygous transgenic lines stably expressing the *Pinb* gene (6).

The original protocol for BABs preparation involved sonication of the sodium alginate emulsion, which produced BABs with diameters ranging from 0.1 to 15 μm with more than 90% of BABs having less than 10 μm in diameter (1). However, transformation efficiency was not high at 0.22%. Efficiency of transient transformation with BABs was improved by the application of electroporation (7) and lipofection technique (4). Further improvement of the BABs transformation method is the fabrication of a bead-maker set-up that allows the production of uniform BABs.

In this chapter, the protocol for BABs-mediated transformation of rice with large DNA fragments will be introduced, then the improved method for BABs-mediated transformation in tobacco BY-2 cells using a bead-maker system will be described.

2. Materials

2.1. BABs-Mediated Transformation of Rice with Large DNA Fragments

2.1.1. Establishment of Suspension Cell Cultures

1. Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare).
2. LSD2G medium: Linsmaier and Skoog (LS) medium (8) supplemented with 2 mg/L 2,4-D and solidified with 0.25% gellan gum. Autoclave and store at 4°C.
3. mN6S medium: N_6 medium (9) supplemented with 0.025 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25 mg/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.025 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, MS vitamins, 1 mg/L 2,4-D, and 30 g/L sucrose, pH 5.8. Autoclave and store at 4°C.
4. Sodium hypochlorite.
5. Petri dish (60 mm $\times$ 15 mm) (BD Falcon, NJ, USA).

2.1.2. Preparation of BABs with BAC DNA

1. Sodium alginate solution: 0.5% sodium alginate (100–150 cP, Wako Pure Chemical, Osaka, Japan). Autoclave and store at room temperature (see Note 1).
2. Isoamyl alcohol.
3. BAC DNA: 10 μg for one sample (see Note 2).
4. 100 mM CaCl_2 solution. Autoclave and store at room temperature.
5. Ultrasonic disrupter (UR-20P, Tomy Seiko, Tokyo, Japan).

2.1.3. Isolation of Protoplasts from Rice Callus

1. Enzyme solution: 1% Cellulase Onozuka RS (Yakult Honsha Co., Ltd., Tokyo, Japan), 0.1% Pectolyase Y-23 (Kyowa Chemical Products Co., Ltd., Osaka, Japan), and 0.4 M mannitol, pH 5.5. Filter sterilize and store at -20°C .

2. Mannitol solution: 0.4 M mannitol, pH 5.5. Autoclave and store at room temperature.
3. W5 solution: 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 5 mM glucose, pH 5.6. Autoclave and store at room temperature (10).
4. Dropper (Eiken Chemical Co., Ltd., Tokyo, Japan).
5. 15-mL centrifuge tube (round bottom). Autoclave.
6. 30-μm Nylon mesh set on a glass funnel. Autoclave.
7. Hemocytometer.

2.1.4. Transformation

1. MaMg solution: 15 mM MgCl₂, 0.4 M mannitol, and 0.1% MES, pH 5.6. Autoclave and store at room temperature.
2. 40% PEG CMS6: 40% (*w/v*) PEG 6000, 0.4 M mannitol, and 0.1 M Ca(NO₃)₂·4H₂O, pH 7–9. Filter sterilize using a 0.45-μm pore filter and store at –20°C.
3. 0.2 M CaCl₂ solution: 0.2 M CaCl₂ and 0.4 M mannitol, pH 5.8. Autoclave and store at room temperature.
4. Wheat cultured cells HY-1 (*Triticum aestivum* cv. Haruyutaka) (see Note 3).
5. Liquid R2P medium: R₂ medium (11) supplemented with 100 mg/L inositol, 2 mg/L 2,4-D, and 137 g/L sucrose, pH 6.0. Filter sterilize and store at 4°C.
6. R2P medium for immobilization of protoplasts: liquid R2P medium solidified with 2.5% (*w/v*) Seaplaque® agarose (Lonza, Switzerland). Filter sterilize and store at 4°C.

2.1.5. Selection and Regeneration of Transgenic Rice Plants

1. N6AS medium: N₆ medium supplemented with 100 mg/L myo-inositol, 2 mg/L 2,4-D, and 3% (*w/v*) sucrose and solidified with 0.3% agarose type I, pH 6.0. Autoclave and store at 4°C.
2. Regeneration medium: LS medium supplemented with 3% sucrose, 3% sorbitol, 2 g/L casamino acid, 1 g/L 2-(*N*-morpholino) ethanesulfonic acid (MES), 2 mg/L NAA, and 1 mg/L kinetin and solidified with 0.4% gellan gum, pH 5.8. Autoclave and store at 4°C.
3. LS hormone-free medium: LS medium supplemented with 3% sucrose and solidified with 0.25% gellan gum, pH 5.8. Autoclave and store at 4°C.
4. 50 mg/mL hygromycin stock solution. Filter sterilize and store at –20°C.
5. Plant box (60 mm × 60 mm × 100 mm) (Rikagaku Kikai, Co., Ltd., Tokyo, Japan).

2.2. Improved BABs-Mediated Transformation Using a Bead-Maker

2.2.1. Constructing the Bead-Maker

1. Microsyringe pump (MSP-RT, AS ONE, Osaka, Japan).
2. Gastight syringe, 100 μL (Hamilton, NV, USA).
3. Silica capillary tube, 30 μm ϕ (GL Sciences, Tokyo, Japan).
4. Loudspeaker, 4 Ω (FR 8, Visaton, Germany).
5. Sine wave sound generator (AG-203D, Kenwood, Tokyo, Japan).
6. The bead-maker is composed of two major parts: the microsyringe part and vibrator part. The former part is assembled by putting a syringe on a microsyringe pump where the solution flow rate could be accurately controlled. The latter part consists of a loudspeaker which is attached to a moveable rod, fixed to a wooden board and connected to a sine wave sound generator. The frequency and amplitude of the sine wave sound generator are changeable. As a result, vibration of the moveable rod as well as a capillary tube linked between microsyringe part and vibrator part are controllable (Fig. 1).

2.2.2. Preparation of BABs with Uniform Size

1. Sodium alginate solution: 1% (*w/v*) 100–150 cP sodium alginate (Wako, Cat. No. 192-09951) in water. Autoclave and store at room temperature.
2. 0.5 $\mu\text{g}/\mu\text{L}$ pUC18-sGFP (provided by Dr. Yasuo Niwa and Dr. Ikuo Nakamura, University of Shizuoka, Japan).
3. Isoamyl alcohol (3-methyl-1-butanol 98%, SIGMA M32658).
4. Isopropyl alcohol (SIGMA I9030).

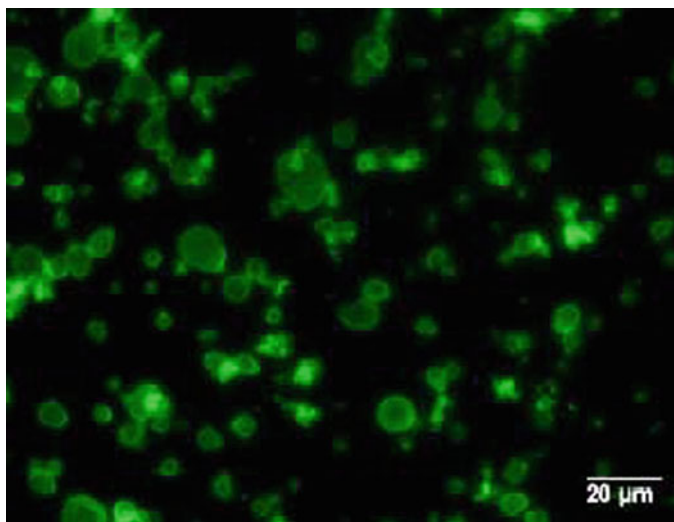


Fig. 1. Bioactive beads stained with DNA-specific fluorescent dye YOYO-1. Bar: 20 μm .

5. 0.1 M CaCl_2 (aq.). Autoclave and store at room temperature.
6. Stock solution of 1:1 (v/v) of 0.1 M CaCl_2 solution and isopropyl alcohol: Prepare 10 mL stock solution by mixing 5 mL of 0.1 M CaCl_2 with 5 mL of isopropyl alcohol. Mix before use.
7. Solidifying solution: freshly prepare before use by first adding 750 μL of CaCl_2 and isopropyl alcohol solution into a 1.5-mL microcentrifuge tube, then add 750 μL of isoamyl alcohol.

2.2.3. Tobacco BY-2 Culture and Protoplast Isolation

1. Tobacco BY-2 cell line (provided by the Experimental Plant Division, Bioresource Center, RIKEN, Japan).
2. 5% (w/v) 2-(*N*-Morpholino)-ethanesulfonic acid (MES) solution: 5% (w/v) MES (Nacalai Tesque, cat. no. 21623-26) in water. Adjust pH to 5.8 with KOH. Autoclave and store at 4°C.
3. 100× KH_2PO_4 solution: 20 g/L KH_2PO_4 in water. Store at 4°C.
4. Tobacco BY-2 culture medium (modified Linsmaier and Skoog medium): 4.3 g/L Murashige and Skoog basal salts mixture (MS salts, Duchefa Biochemie, product no. M0221), 0.05% (w/v) MES solution, 3% (w/v) sucrose, 100 mg/L inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D, and 1× KH_2PO_4 (0.2 g/L). Autoclave and store up to 1 month at room temperature.
5. Enzyme solution: 1% (w/v) cellulase ONOZUKA RS, 0.1% (w/v) Pectolyase Y-23, and 0.4 M mannitol (pH 5.5). Sterilize using 0.2- μm filter and store at -20°C until use.

2.2.4. DNA Transformation

1. 0.4 M mannitol (pH 5.5). Autoclave and store at room temperature.
2. MaMg solution: 0.1% (w/v) MES (pH 5.8), 15 mM MgCl_2 , and 0.4 M mannitol. Autoclave and store at room temperature.
3. PEG solution: 0.4 M mannitol, 40% (w/v) PEG 6000 (Wako), 0.1 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, pH 7–9, and sterilize using 0.45- μm filter. Store at -20°C until use. Thaw the solution at 60°C and cool down to room temperature at least 10 min before use.
4. 0.2 M CaCl_2 solution: 0.4 M mannitol and 0.2 M CaCl_2 . Adjust pH to 5.8 and autoclave. Store at room temperature.
5. W5 solution: 154 mM NaCl, 125 mM CaCl_2 , 5 mM KCl, and 5 mM glucose. Adjust pH to 5.6. Autoclave and store at room temperature.
6. Tobacco BY-2 protoplast medium: 4.3 g/L Murashige and Skoog basal salts mixture (MS salts, Duchefa Biochemie), 0.05% (w/v) MES solution from stock solution, 1% (w/v) sucrose, 100 mg/L inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D and 1× KH_2PO_4 (0.2 g/L), and 0.4 M mannitol (pH 5.5). Autoclave and store at room temperature.

3. Methods

3.1. BABs-Mediated Transformation of Rice with Large DNA Fragments

Transformation of large DNA fragments into plants is a promising strategy to improve complicated agronomic properties. This strategy will make it possible to introduce multiple genes into plants at once, thus it would expand the utilization of plant genetic engineering. However, it is still difficult to transfer large DNA fragments into plants using conventional methods. The protocol described here is a novel transformation method, using BABs, for transfer of large DNA fragments into rice protoplasts. One of the problems in using large DNA molecules for plant transformation is fragmentation caused by physical damage. However, with the BABs method, this problem is overcome by immobilizing large DNA fragments in the beads. The immobilization protects large DNAs from physical damage, thus making these DNA fragments stable during transformation experiments. Thus, it may represent a good method to transfer large DNA fragments into plant cells.

The BABs transformation method utilizes polyethylene glycol to introduce DNA into plant protoplasts. The establishment of a good quality suspension cell culture and isolation of protoplasts in excellent condition are critical points for this transformation. Rice has been successfully transformed with ca. 100 kb of BAC DNA using the protocol described below. The tissue culture of rice is based on the report of Otani et al. (12), and the transformation protocol is based on the paper by Wada et al. (6). The transformation using BABs is also reviewed in refs. 13, 14.

3.1.1. Establishment of Suspension Cell Cultures

1. Remove husks from rice seeds and soak them into sodium hypochlorite solution for 1 h.
2. Wash seeds with sterilized distilled water five times.
3. Put seeds on LSD2G medium.
4. Incubate them at 26°C under a 16-h light/8-h dark photoperiod.
5. After about 1 month, pick up the formed calli and transfer them into 30 mL of mN6S medium in 100-mL flask covered with aluminum foil.
6. Culture them on a rotary shaker at 120 rpm at 26°C under a 16-h light/8-h dark photoperiod.
7. Subculture two spoonfuls of suspension calli into a new mN6S medium every week (see Note 4).

3.1.2. Preparation of BABs with BAC DNA

1. Add 900 μ L of isoamyl alcohol to 100 μ L of sodium alginate solution in a 1.5-mL Eppendorf tube (tube 1).
2. Put 450 μ L of 100 mM CaCl_2 into another 1.5-mL Eppendorf tube (tube 2) and add 10 μ g of BAC DNA (50 μ L) into tube 2. Mix gently.

3. Emulsify the solution in tube 1 using an ultrasonic disrupter set to maximum power for 10 s.
4. Immediately add 500 μL of CaCl_2 solution containing BAC DNA into the emulsified solution.
5. Mix the solution gently but thoroughly for 1 min.
6. Centrifuge at $2,300\times g$ for 5 min.
7. Remove the upper isoamyl alcohol phase without removing the beads located around the interface.
8. Add 100 mM CaCl_2 solution into the lower phase solution, then mix gently.
9. Centrifuge at $2,300\times g$ for 5 min.
10. Carefully remove the solution making sure not to disturb the precipitated beads.
11. Add 100 mM CaCl_2 solution into the tube.
12. Centrifuge at $2,300\times g$ for 5 min.
13. Repeat steps 10–12 three times.
14. Suspend the precipitate in 50 μL of 100 mM CaCl_2 solution. Figure 1 shows the BABs observed under the microscope (see Note 5).

*3.1.3. Isolation
of Protoplasts
from Rice Callus*

1. Remove the liquid medium from 3- to 4-day-old suspension cell culture by tilting the flask.
2. Transfer two spoonfuls of calli into 5 mL of enzyme solution in a Petri dish (60 mm $\times$ 15 mm).
3. Seal Petri dish with Parafilm and shake at 30 rpm at 30°C for 3 h.
4. Incubate at 30°C for 1 h without shaking.
5. Check the condition of protoplasts under the microscope. If many round protoplasts have already been produced, stop the incubation and go to the next step. If the digestion of cell wall is still not enough, incubate for another hour.
6. Pour the protoplast solution to a 30- μm nylon mesh set on a glass funnel and collect the filtered solution in a 15-mL centrifuge tube.
7. Fill the 15-mL centrifuge tube with W5 solution slowly until the 15-mL mark and mix gently using a dropper (see Note 6).
8. Centrifuge at $130\times g$ for 4 min.
9. Remove enzyme solution using a dropper leaving ca. 1 mL solution in the tube.
10. Suspend the protoplasts by shaking gently.
11. Add W5 solution slowly along the inner side of the tube and mix gently using a dropper.
12. Centrifuge at $130\times g$ for 4 min.

13. Remove solution using a dropper leaving ca. 1 mL solution in the tube. If multiple tubes with the same sample exist, pool them together into one tube at this step.
14. Add W5 solution slowly along the inner side of the tube and mix gently using a dropper.
15. Centrifuge at $130\times g$ for 4 min.
16. Remove solution using a dropper leaving ca. 1 mL solution in the tube.
17. Suspend the protoplasts gently.
18. Measure the cell density using a hemocytometer and calculate the volume of solution necessary to adjust the cell density to 2×10^6 cells/mL.
19. Add W5 solution slowly along the inner side of the tube and mix gently using a dropper.
20. Centrifuge at $130\times g$ for 4 min.
21. Remove the solution gently using a dropper.
22. Add MaMg solution into the precipitate to adjust the cell density to 2×10^6 cells/mL.

3.1.4. Transformation

1. Transfer 500 μ L protoplast suspension of 2×10^6 cells/mL into a 15-mL glass centrifuge tube.
2. Add 50 μ L of the solution containing BABs with BACs into the protoplast suspension and mix gently.
3. Add 825 μ L of 40% (*w/v*) PEG CMS6 solution into the solution (final concentration of PEG is 24%) and mix gently by inverting the tube 4–6 times.
4. Incubate the tube horizontally positioned at room temperature for 10 min.
5. Add 815 μ L of 0.2 M CaCl_2 solution to dilute the PEG solution and mix gently by inverting the tube 4–6 times to disperse the protoplasts.
6. Add 815 μ L of 0.2 M CaCl_2 solution twice and mix gently by inverting the tube 4–6 times.
7. Repeat step 6.
8. Fill the tube with W5 solution until the 15 mL mark.
9. Centrifuge at $130\times g$ for 3 min.
10. Remove the solution gently without disrupting the precipitates using a dropper.
11. Wash the precipitated protoplasts with W5 solution again.
12. Suspend the protoplasts into 1 mL of R2P medium.
13. Mix the protoplast solution with an equal volume of pre-warmed R2P medium containing 2.5% (*w/v*) Seaplaque® agarose using a dropper (see Note 7).

14. Using a dropper, transfer the agarose mixture into a Petri dish that had been stored at -20°C (see Note 8).
15. Let the agarose block solidify on the cold Petri dish.
16. Add 5 mL of liquid R2P medium and wheat HY-1 cells into the Petri dish (60 mm $\times$ 15 mm) in which solidified agarose blocks containing protoplasts are placed.
17. Seal the Petri dish with Parafilm.
18. Incubate the Petri dish with shaking at 30 rpm at 26°C in the dark for 2 weeks.

3.1.5. Selection and Regeneration of Transgenic Rice Plants

1. Transfer the agarose blocks to liquid R2P medium containing 50 $\mu\text{g}/\text{mL}$ hygromycin in order to select for transformed colonies.
2. After 14 or 28 days, transfer the agarose blocks containing colonies of at least 1 mm in diameter to solidified N₆AS medium (Fig. 2).
3. Incubate them at 26°C under a 16-h light/8-h dark photoperiod for 2–4 weeks until calli grow well.
4. Transfer the hygromycin-resistant callus to regeneration medium containing 50 $\mu\text{g}/\text{mL}$ hygromycin.
5. Culture them at 26°C under a 16-h light/8-h dark photoperiod.
6. Transfer the regenerated callus that formed shoots and roots to LS hormone-free medium supplemented with 50 $\mu\text{g}/\text{mL}$ hygromycin in a plant box.
7. Incubate them at 26°C under a 16-h light/8-h dark photoperiod until the transgenic shoots reach to the cover of the container.

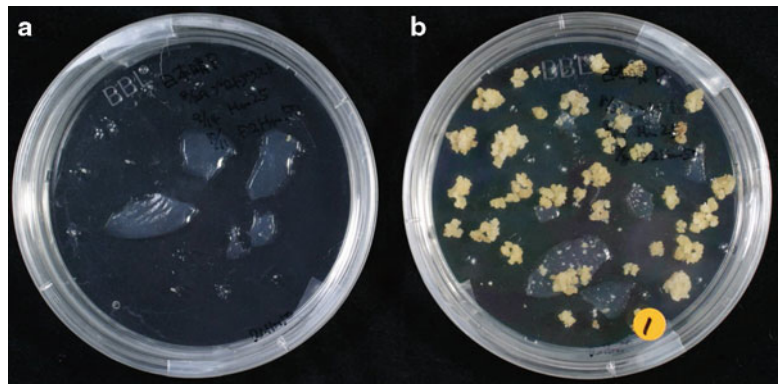


Fig. 2. Selection of calli with hygromycin on N₆AS medium. (a) Control. (b) Transgenic calli. (With kind permission from Springer Science + Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750–68, Wada, N., Kajiyama, S., Akiyama, Y., Kawakami, S., No, D., Uchiyama, S., Otani, M., Shimada, T., Nose, N., Suzuki, G., Mukai, Y., Fukui, K., fig. 2).

8. Open the cover a little to allow shoots to become acclimated to the outside environment, and then incubate for 1 day.
9. Transfer the plants to pots and grow them in an incubator or greenhouse at 26°C.

3.1.6. Molecular Analysis of Transgenic Rice

Molecular analysis of transgenic rice is necessary to confirm the presence of transgenes and reveal their structures of introduced fragments in transgenic plants. PCR analysis is suitable to check the presence of transgenes in the early stage of regeneration of transgenic plants because it does not need much amount of plant materials. Southern blot analysis is also a useful method to confirm the presence of transgenes and is required to check the copy number of transgenes in transgenic plants. By choosing proper probes, it will be possible to estimate the intactness of introduced fragments. FISH analysis is also an attractive method. Because the size of introduced DNA fragments is large in this experiment, signals can be easily detected by FISH analysis compared with in case of transgenic plants with a single gene (15, 16). FISH analysis can visualize the location of transgene integration and identify the homozygous transgenic plant faster than segregation analysis (Fig. 3). It also can be used to study the intactness of introduced DNA fragments (17). The molecular analyses in RNA and protein level such as RT-PCR, Northern blot, and Western blot analyses are

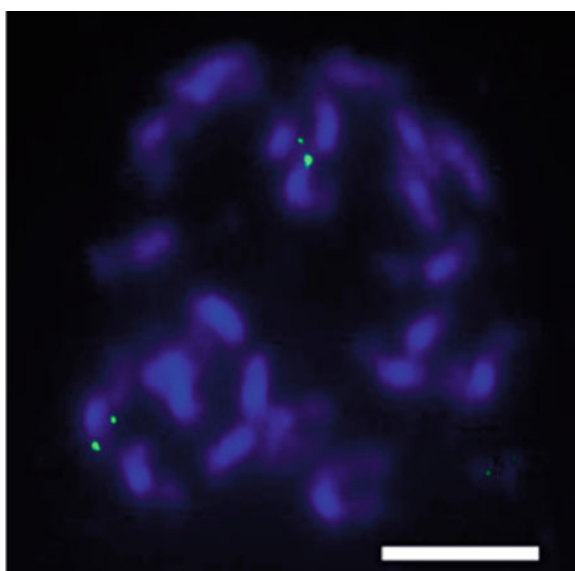


Fig. 3. FISH analysis of the T_2 transgenic plant. The two paired green signals indicate the integration sites of pBI BAC 10–60. The signals also show that transgenes are integrated into the telomeric region and that this transgenic plant is homozygous. Bar: 10 μ m. (With kind permission from Springer Science + Business Media: Plant Cell Reports, Bioactive beads-mediated transformation of rice with large DNA fragments containing *Aegilops tauschii* genes, 28, 2009, 750–68, Wada, N., Kajiyama, S., Akiyama, Y., Kawakami, S., No, D., Uchiyama, S., Otani, M., Shimada, T., Nose, N., Suzuki, G., Mukai, Y., Fukui, K., fig. 4).

also required to identify the most prominent transgenic plants depending on the introduced genes (6).

3.2. Improved BABs-Mediated Transformation Using a Bead-Maker

Using the conventional method for BABs preparation (1–7, 13, 14) (i.e., using a sonicator), the CaCl_2 solution containing plasmid DNA was added to alginate droplets emulsified in isoamyl alcohol-alginate emulsion solution, which consequently resulted in immobilization of DNA mainly on the surface of BABs. This might be the cause of inefficient transfer of DNA to plant protoplasts due to ineffective immobilization of DNA on BABs. Furthermore, various sizes and shapes of beads produced by the sonication method were considered to give low transformation efficiency. Thus, a bead-maker system for BABs production, which enables more efficient immobilization of DNA and allows bead size control has been developed. Using the new system, alginate was mixed with plasmid DNA and then subjected to the bead-maker to produce BABs. Then, all the DNA molecules were entrapped both within the BABs and on the surface of BABs. Moreover, it was found that the size of BABs produced by the bead-maker was controllable by adjustment of solution flow rate as well as the amplitude and frequency of the sine wave sound generator. The transformation efficiency of BY-2 cells was evaluated using various sizes of BABs, and it was found that the size of beads had an effect on transient transformation efficiency in tobacco BY-2 cells. BABs 1–6 μm in diameter are suitable for obtaining higher transformation efficiency of up to 10%.

3.2.1. Preparation of Uniform BABs

1. Prepare DNA-containing alginate solution by mixing 100 μL sodium alginate solution (1% w/v) with 100 μL pUC18-sGFP (0.5 $\mu\text{g}/\mu\text{L}$) and carefully load the freshly prepared solution in 100 μL syringe. Connect the syringe to the capillary tube and place the syringe at microsyringe pump as shown in Fig. 4.

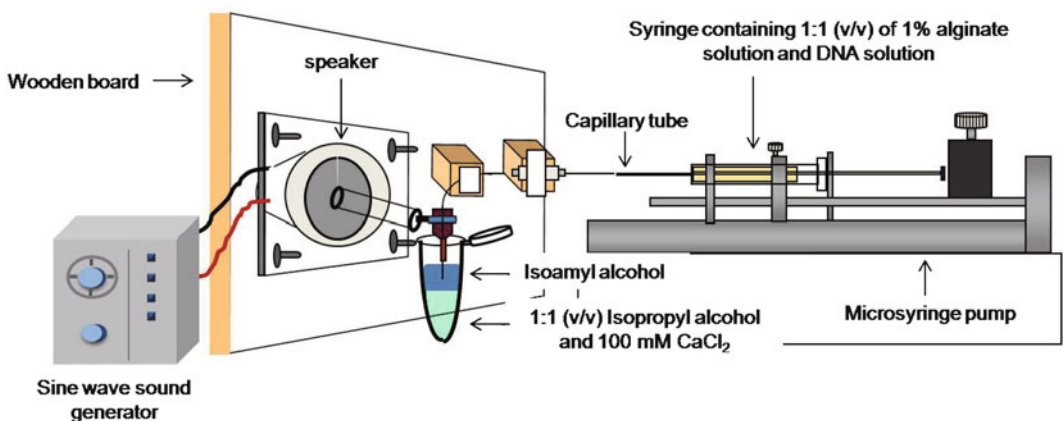


Fig. 4. Schematic diagram of the bead-maker assembly, showing the microsyringe part which controls the solution flow rate, and vibration control part which regulates the vibration of capillary tube and consequently drop the solution to bead-solidifying solution.

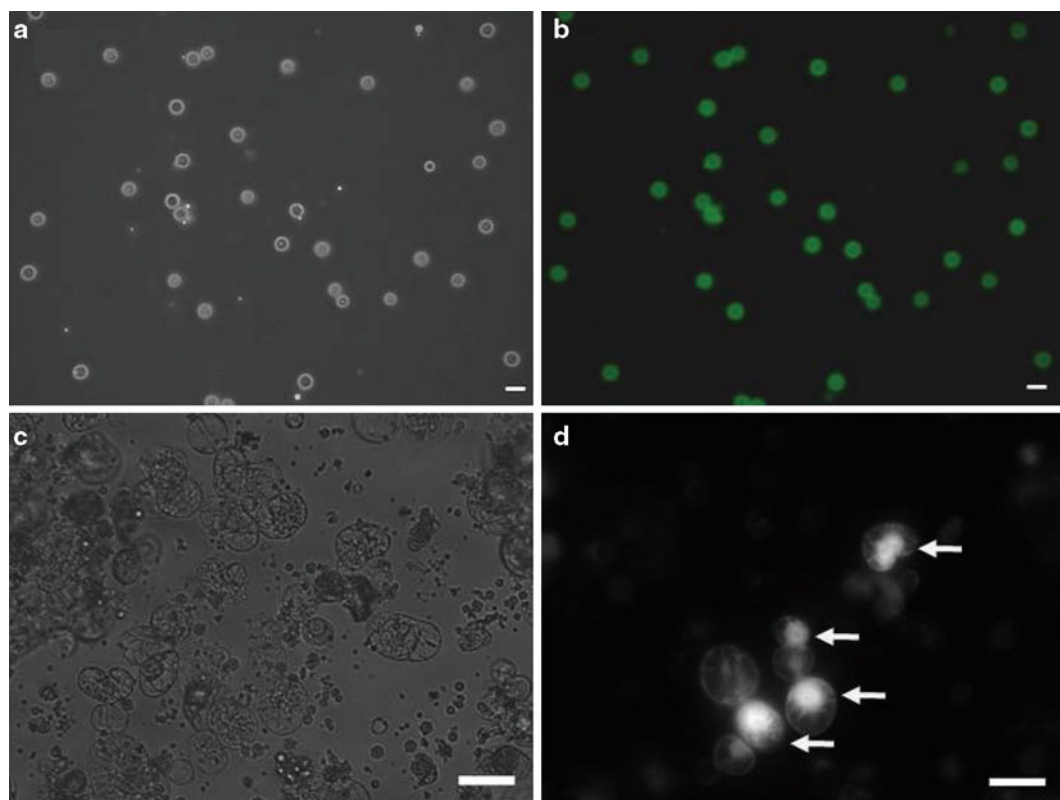


Fig. 5. Bioactive beads prepared using the new bead-maker. (a) Phase-contrast and (b) fluorescent image. Tobacco BY-2 protoplasts after 48 h transformation, (c) phase-contrast image and (d) fluorescent image under GFP filter. White arrows indicate GFP-expressing protoplasts. Bars: a, b: 10 μm and c, d: 20 μm .

2. Set the solution flow rate of microsyringe pump to 0.4 $\mu\text{L}/\text{min}$ and adjust the frequency and amplitude of sine wave sound generator to 250 Hz and to 10 (arbitrary unit set at the sine wave sound generator), respectively (see Note 9).
3. Put the 1.5-mL microcentrifuge tube of solidifying solution in the plastic rack and set as shown in Fig. 4.
4. Switch on power to the microsyringe pump and sine wave sound generator. While the bead preparation machine is working, DNA-containing alginate solution is pumped out in a constant flow rate. Simultaneously, the sine wave sound generator produces sound wave at the speaker and results in vibration of the moveable rod connected to the speaker. Consequently, vibration of the capillary tube linked to the moveable rod drops alginate-DNA solution to the solidifying solution. Isoamyl alcohol keeps the droplets spherical, and CaCl_2 -isopropyl alcohol solution solidifies the alginate-DNA droplets. Size of beads prepared by this method is approximately 1–6 μm (Fig. 5a, b).

5. Collect DNA-immobilized beads by centrifugation at $2300 \times g$ for 5 min and wash the beads three times with 0.1 M CaCl_2 .
6. Resuspend DNA-immobilized beads in 0.1 M CaCl_2 (50 μL) and use for transformation.

3.2.2. Tobacco BY-2 Culture and Protoplast Isolation

1. Culture tobacco BY-2 cells in BY-2 culture medium at 26°C in the dark with 120 rpm shaking. Subculture tobacco BY-2 cells once a week into 100 mL fresh culture medium.
2. Take 30 mL of 3 or 4-day cultured tobacco BY-2 cells to 50-mL falcon tube and centrifuge at $160 \times g$ for 5 min.
3. Wash the cells with 0.4 M mannitol solution and centrifuge at $160 \times g$ for 5 min.
4. Collect cell pellet and add 15 mL enzyme solution.
5. Incubate the solution at 30°C for 1 h and 30 min or until cell wall is completely digested. Gently mix cell enzyme suspension every 15 min to disperse the precipitated cells.
6. Check protoplasts under the microscope. If cell wall is completely digested and protoplasts become single and round, collect protoplasts by centrifuge at $160 \times g$ for 5 min.
7. Wash the protoplasts twice with 30 mL of 0.4 M mannitol solution.
8. After collecting the protoplasts by centrifugation, resuspend protoplasts at $2 \times 10^6/\text{mL}$ in MaMg solution. Estimate the protoplast concentration by using a hemocytometer. Use these protoplasts for transformation.

3.2.3. DNA Transformation and Observation of GFP Expression

1. Transfer 500 μL protoplast suspension using dropper into a 15 mL round-bottom glass tube and add 50 μL DNA-immobilized beads solution.
2. Slowly add 825 μL of 40% PEG solution (to get 24% final concentration) into the protoplast and beads suspension, then gently and thoroughly mix by swirling.
3. Let the transformation mixture stand at room temperature for 30 min.
4. Slowly add 815 μL of 0.2 M CaCl_2 or W5 solution and mix gently by inverting 4–6 times.
5. Add 1,630 μL of 0.2 M CaCl_2 or W5 solution slowly and mix gently by inverting 4–6 times.
6. Repeat step 5.
7. Centrifuge at $160 \times g$ for 5 min and wash again using W5 solution.
8. Collect the protoplast pellet by centrifugation, then carefully resuspend the protoplasts in 1 mL tobacco BY-2 protoplast

medium. Transfer the protoplast suspension into a 3.5-mm glass-bottom dish and seal the disc with Parafilm.

9. Incubate the protoplasts at 26°C in the dark for 2 days.
10. Perform observation of GFP protein expressed from tobacco BY-2 protoplasts using an inverted fluorescence microscope (Olympus IX81) through a fluorescence filter WIB/GFP (Fig. 5c, d).

4. Notes

1. Since alginate is a natural product purified from brown algae, its physico-chemical nature depends on the place of production and the type of brown algae used. We found that the kind of sodium alginate used affected transient transformation efficiency. The sodium alginate which had the lower viscosity produced higher transient transformation efficiency. The best sodium alginate may be different depending on plant species to be used.
2. pBI BAC 10–60 was kindly provided by Prof. Mukai (Osaka Kyoyiku University, Japan) (18) for transformation. The size of the pBI BAC 10–60 is ca. 100 kb. Ten microgram was enough for our experiment, but if the larger size of BAC DNA is used for transformation, it will be necessary to increase the amount of DNA used.
3. Cultured cells of wheat line HY-1 (*Triticum aestivum* cv. Haruyutaka) were kindly provided by Profs. T. Shimada and M. Otani (Ishikawa Prefectural University, Japan) and used as nurse cells to help the growth of protoplasts. The effectiveness of HY-1 cell as nurse cells was investigated by Profs. T. Shimada and M. Otani (personal communication).
4. Establishment of good suspension cell cultures is an important step for this experiment. If the calli do not grow well or turn brown during the culture, that cell line should be discarded. It is recommended to always make several cell lines and use the best one for the experiment.
5. It is very important to use good plant materials in this experiment. If there are not sufficient protoplasts at this stage, it may be because of the condition of suspension cell culture or the difference in plant species and/or varieties. It is recommended to have several lines of suspension cell culture and use the best line for the experiment.
6. During this experiment, it is important to handle the solution containing protoplasts gently. The protoplasts must be suspended and mixed very gently and carefully using a dropper.

7. After pre-warming the solidified R2P medium to melt it, the medium should be cooled down below 60°C.
8. One milliliter of agarose mixture was dropped onto the cooled Petri dish to make one agarose block.
9. It was found that the viscosity of sodium alginate and the alginate-DNA solution would affect the size of BABs. The results reported here were obtained using sodium alginate with 100–150 cP (Wako, Cat. No. 192-09951). When sodium alginate with a different viscosity or a different ratio for the amount of alginate and DNA in solution were used, varied sizes of BABs were obtained, even though the same settings for solution flow rate, amplitude, and frequency of sine wave sound generator were used. Therefore, it is recommended to optimize the conditions (solution flow rate, amplitude, and frequency of sine wave sound generator) for BABs preparation when a different sodium alginate solution is used. The same is true once the concentration of DNA is varied relative to sodium alginate. In our case, we used 1:1 (*v/v*) of 1% alginate solution and 0.5 µg/µL of pUC18-sGFP (4.1 kb). If a higher molecular weight of DNA vectors is used, higher settings for amplitude and frequency of the sine wave sound generator can be used to get the suitable sizes of BABs (1–6 µm) for transformation.

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Part IV

Transformation Technology: Cereals and Other Monocots

Chapter 10

***Agrobacterium*-Mediated Transformation of *Sorghum bicolor* Using Immature Embryos**

Songul Gurel, Ekrem Gurel, Tamara I. Miller, and Peggy G. Lemaux

Abstract

Successful efforts describing in vitro culturing, regeneration, and transformation of grain sorghum were reported, using particle bombardment, as early as 1993, and with *Agrobacterium tumefaciens* in 2000. Reported transformation efficiencies via *Agrobacterium* routinely range from 1 to 2%. Recently, such efficiencies via *Agrobacterium* in several plant species were improved with the use of heat and centrifugation treatments of explants prior to infection. Here, we describe the successful use of heat pretreatment of immature embryos (IEs) prior to *Agrobacterium* inoculation to increase routine transformation frequencies of a single genotype, P898012, to greater than 7%. This reproducible frequency was calculated as numbers of independently transformed IEs, confirmed by PCR, western, and DNA hybridization analysis, that produced fertile transgenic plants, divided by total numbers of infected IEs.

Key words: *Agrobacterium tumefaciens*, GFP, Heat treatment, Immature embryos, Phosphomannose isomerase, Sorghum, Transformation

1. Introduction

Worldwide sorghum (*Sorghum bicolor* (L.) Moench) is an important cereal crop, ranking fifth in production. Its relative tolerance to drought and heat makes it an ideal grain for human and animal consumption in areas with extreme temperatures and minimal precipitation, like the semiarid regions of Asia and Africa. Use of dry-land crops, such as sorghum, will surely increase elsewhere as global climate changes lead to dramatic changes in temperatures and water availability. With these likely changes, efforts to increase sorghum's yield and quality will be of critical importance.

Application of genetic engineering and genomic technologies is one means to improve sorghum in the future. Successful in vitro culturing, regeneration, and transformation of grain sorghum have been reported (1–15). The first fertile transgenic sorghum plants

were obtained in 1993, using particle bombardment (3), and in 2000 by *Agrobacterium*-mediated transformation (14). Several studies have focused on improving transformation and regeneration frequencies via either strategy (6, 7, 10, 16, 17); reported transformation efficiencies via *Agrobacterium* were in the range of 1–2%. In a 2006 study (10), a 4.5% frequency (4 events from 89 explants) was achieved in 1 of 17 experiments; however, frequencies in the other 16 ranged from 0.3 to 1.9%. Results like these underscore the recalcitrance and lack of reproducibility of such approaches with sorghum (18, 19).

Transformation efficiencies via *Agrobacterium* were improved recently in several plant species using heat and centrifugation treatments of transformation explants. For example, Khanna et al. (20) reported heat shocking banana suspension cells before *Agrobacterium* infection, doubling postinfection viability and increasing transformation efficiency; centrifuging suspension cells with *Agrobacterium* resulted in quadrupled efficiencies. In 2006 rice and maize transformation frequencies were increased several-fold by heat treatment and centrifugation of immature embryos (IEs) before *Agrobacterium* infection (21). The increased transformation frequencies reported in Gurel et al. (8) suggest that heat pretreatment of IEs prior to infection of IEs from sorghum variety P89012 induces a stress response that allows cells to survive cocultivation with *Agrobacterium*. In addition, according to Carvalho et al. (16), as well as from our efforts aimed at transforming several sorghum varieties, one primary factor affecting success of *Agrobacterium*-mediated transformation is the cocultivation method used to infect IEs.

Using a selectable marker gene for phosphomannose isomerase (*pmi*) and an *sgfp* reporter gene, a relatively efficient, reproducible protocol for *Agrobacterium*-mediated transformation of P898012 was developed using heat treatment of IEs prior to *Agrobacterium* infection (8). Evaluation of effects of heat treatment and centrifugation on transient GFP expression and stable transformation frequency is described here.

2. Materials

2.1. Growth of Donor Plants

1. Sorghum seeds of P898012 can be obtained from the USDA ARS Germplasm Resources Information Network (<http://www.ars-grin.gov/npgs/orders.html>).
2. Use 4-gallon pots containing Supersoil potting soil (Rod McClellan Co., South San Francisco, CA, USA).
3. Grow either in walk-in growth chamber (Kolpak, River Falls, WI, USA) with 16 h day/8 h night temperatures of 28/22°C

and sodium vapor and metal halide lights at $\sim 700 \mu\text{mol/s/m}^2$ or in the greenhouse under comparable natural and artificial lighting and day/night temperatures.

4. Use Jack's Professional Fertilizer 20-20-20 (J.R. Peters Inc., Allentown, PA, USA).

2.2. Sterilization of Plant Seeds: Isolation of Immature Embryos

1. Panicles ~ 12 – 14 -day postpollination.
2. 70% ethanol.
3. 20% (v/v) bleach solution (5.25% sodium hypochlorite).
4. Sterile distilled water.
5. Sterile 7 cm diameter Whatman #1 filter disks.
6. Liquid inoculation medium (see Table 1).

2.3. Pretreatment with Heat

1. Carefully controlled 43°C water bath.
2. Water bath at 25°C .

2.4. Introduction of Plasmid Vectors into *Agrobacterium*

1. Binary vector, pPZP201-GFP-PMI (pGFP-PMI; see Fig. 1) (7), containing *sgfp* encoding the green fluorescence protein (GFP); (22) and *pmi* encoding phosphomannose isomerase (23).
2. *A. tumefaciens* strains, EHA101 (24) and LBA4404 (25).

2.5. Preparation of *Agrobacterium* Suspension and Infection of IEs

1. Master plate of *Agrobacterium* with pPZP201-GFP-PMI on YEP agar medium (see Note 1) plus 100 mg/L spectinomycin (Sigma S4014) for EHA101 and LBA4404.
2. Liquid inoculation medium (see Table 1).
3. Liquid culture of *Agrobacterium* at $\text{OD}_{600} = 0.4$ (see Subheading 3.4).
4. Stock of 200 mM acetosyringone (filter sterilized; Sigma D134406).

2.6. Culturing of Tissue, Selection, and Regeneration

1. Various tissue culture media (see Table 1).
2. Full-strength Murashige and Skoog (MS) basal salts (Phytotech Labs: M419) and MS micronutrient solution (Phytotech Labs: M654) are used except in rooting medium.
3. Phytigel (Sigma: P8169) at 2.0 g/L is added to all media before autoclaving at 121°C .
4. 1% (w/v) polyvinylpyrrolidone (PVP) (Sigma: 234257) added to medium before autoclaving to reduce tissue blackening of high-phenolic cultivars, e.g., P898012.
5. Mannose (Sigma: M6020) is added at 2%, 1.5%, and 1.0% to selection media.
6. After autoclaving, cool media to 55°C before adding carbenicillin (Sigma: C9231), acetosyringone (Sigma: D134406), and ascorbic acid (Sigma: A4544).

Table 1 Chemical composition of different culture media used for tissue culture and transformation studies of sorghum.

Components (stock concentrations)	Inoculation medium (liquid) ^a	Cocultivation medium ^a	Callus induction medium ^a	Selection medium I ^a	Selection medium II ^a	Selection medium III ^a	Regeneration medium ^a	Rooting medium ^a
Murashige and Skoog basal salts	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g	2.2 g
Nicotinic acid (5 mg/mL)	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	50 µL
Pyridoxin HCl (5 mg/mL)	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	50 µL
Thiamine HCl (10 mg/mL)	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	50 µL
Myo-inositol	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g	0.05 g
2,4-Dichlorophenoxy acetic acid (2,4-D) (0.5 mg/mL)	4 mL	4 mL	4 mL	4 mL	4 mL	4 mL	–	–
Sucrose	68.5 g	20 g	30 g	30 g	20 g	15 g	–	–
Glucose	36 g	10 g	–	–	–	–	–	–
Mannose	–	–	–	20 g	15 g	10 g	10 g	10 g
2-(<i>N</i> -morpholino) ethane-sulfonic acid (MES)	0.6 g	0.6 g	–	–	–	–	–	–
Proline	–	0.7 g	–	–	–	–	0.7 g	–

Polyvinylpyrrolidone (PVP)	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g	10 g
Indole acetic acid (IAA) (1 mg/mL)	–	–	–	–	–	–	–	–	200 µL	200 µL
Kinetin (1 mg/mL)	–	–	–	–	–	–	–	–	500 µL	–
1-Naphthaleneacetic acid (NAA) (1 mg/mL)	–	–	–	–	–	–	–	–	–	200 µL
Indole-3-butyric acid (IBA) (1 mg/mL)	–	–	–	–	–	–	–	–	–	200 µL
MS Micronutrient solution	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	–	–
pH ^b	5.6	5.2	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Phytigel	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g
Autoclave for	22 min	22 min	22 min	22 min	22 min	22 min	22 min	22 min	22 min	22 min
Acetosyringone (200 mM)	500 µL	500 µL	–	–	–	–	–	–	–	–
Carbenicillin (100 mg/L)	–	–	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Ascorbic acid (100 mg/mL)	–	100 µL	–	–	–	–	–	–	–	–

^aAmounts listed are for 1 L of medium
^bFor all media, pH adjusted with 1 N KOH

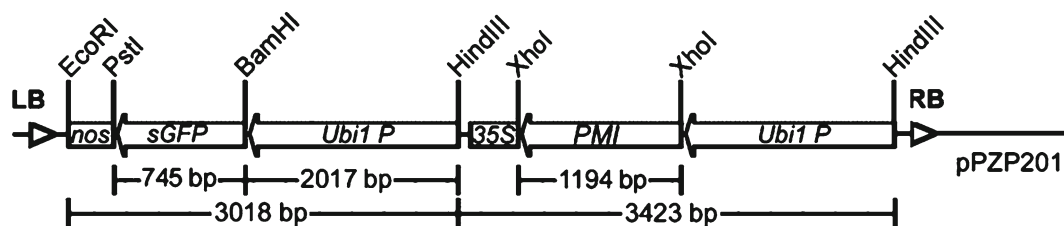


Fig. 1. pGFP-PMI Map (29). *Ubi1 P* ubiquitin promoter and first intron, *35S* and *nos* 3' termination regions, *PMI* phosphomannose isomerase, *sGFP* synthetic green fluorescence protein, physical distances in base pairs (bp), *EcoRI*, *PstI*, *BamHI*, *HindIII*, *XhoI* restriction sites for respective enzymes, *LB*, *RB* left and right borders, respectively, of *Agrobacterium tumefaciens* T-DNA; arrows: direction of transcription of *PMI* and *GFP* genes; pPZP201 vector backbone (30).

7. Stocks of nicotinic acid (Sigma: N0765), pyridoxine HCl (Sigma: P8666), thiamine HCl (Sigma: T3902), and ascorbic acid (Sigma: A4544) are prepared in water. Store at 4°C in 10 mL aliquots.
8. Dissolve 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma: D7299) in 200-proof ethanol; add water for 0.5 mg/mL stock; add to medium before autoclaving. Store at 4°C.
9. Dissolve 3-indole-acetic acid (IAA) (Phytotech Labs: I885) in 200-proof ethanol; add water for 1 mg/mL stock; filter-sterilize. Store at -20°C.
10. Dissolve 1-naphthaleneacetic acid (NAA) (Phytotech Labs: N600) in 1 N KOH; add water for 1 mg/mL stock; filter-sterilize. Store at -20°C.
11. Dissolve indole-3-butyric acid (IBA) (Phytotech Labs: I538) in 200-proof ethanol; add water for 1 mg/mL stock; filter-sterilize. Store at -20°C.
12. Store *N*⁶-furfuryladenine (kinetin) (Sigma: K3253) in sterile 1 mL aliquots at -20°C. Add to medium after autoclaving.
13. Dissolve 3',5'-dimethoxy-3'-hydroxyacetophenone (acetosyringone) (Sigma: D134406) in dimethylsulphoxide (DMSO) (390 mg/10 mL DMSO); filter-sterilize. Store at 4°C.
14. Dissolve carbenicillin (Sigma: C3416) in water for 100 mg/L stock; filter-sterilize. Store at -20°C in aliquots.

3. Methods

3.1. Growth of Donor Plants

1. Plant four seeds per 4-gallon pot and thin to three plants following germination.
2. Keep plants well-watered to avoid water deficit, especially during seed set, and fertilize twice weekly with Jack's Professional Fertilizer 20-20-20 (see Note 2).

3.2. Sterilization of Plant Seeds: Isolation of Immature Embryos

(Steps 3–6 should be done in a laminar flow hood)

1. At ~12–14-day post-pollination, IEs should be ~1.5 mm, just turning opaque (see Notes 3 and 4).
2. Remove seeds from spikes; place in sterile 100 mm×20 mm petri plate.
3. Sterilize in 70% ethanol for 5 min; rinse twice with ddH₂O (see Note 5).
4. Place seeds in sterile 50 mL centrifuge tube; add 40 mL 20% v/v bleach (5.25% hypochlorite). Place on rotary shaker for 20 min to agitate seeds.
5. Rinse seeds in hood in sterile ddH₂O four times for 1 min each; pour off excess liquid; place seeds in sterile petri plate with two sterile, 7 cm Whatman #1 filter papers.
6. Excise IEs (see Note 6) and place in 1.5 mL microfuge tube with room temperature liquid inoculation medium, ~25–30 IEs per tube.

3.3. Pretreatment with Heat

1. Remove inoculation medium leaving only enough to cover IEs.
2. Incubate tube with IEs in carefully controlled 43°C water bath for 3 min.
3. After heat treatment, cool tubes for 2 min in 25°C water bath (see Notes 7 and 8).
4. Remove excess liquid medium from heat-treated IEs.

3.4. Preparation of *Agrobacterium* Suspension

1. Dissolve spectinomycin (Sigma: S4014) in water for 100 mg/mL stock; filter sterilize; store at –20°C in aliquots. Add stock to achieve 100 mg/L final concentration.
2. For glycerol stocks of *Agrobacterium*, grow bacteria from single colony in sterile 14 mL snap cap tube with 5 mL YEP medium (see Note 1) plus 100 mg/L spectinomycin for EHA101 and LBA4404 for 8–12 h at 28°C, shaking at 225 rpm. Following incubation, place 0.75 mL bacterial culture and 0.75 mL sterile 80% glycerol in 1.5 mL microfuge tube, invert to mix and store for up to 1 year at –80°C.
3. Master plate of *A. tumefaciens* EHA101 or LBA4404 with pPMI-GFP is made by streaking from glycerol stock with a sterile inoculating loop onto YEP agar medium+100 mg/L spectinomycin for EHA101 and LBA4404 and cultured at 28°C for 2 days. Master plate can be kept for 2 weeks at 4°C and used to streak additional plates for transformation.
4. One day prior to transformation, use a sterile inoculating loop to streak a single EHA101 or LBA4404 *Agrobacterium* colony

from the master plate onto a fresh YEP plus 100 mg/L spectinomycin agar plate. Incubate ON at 28°C.

5. For transformation, use a sterile metal spatula to resuspend two pencil-eraser-sized portions of the culture from the ON plate in 5 mL liquid inoculation medium (Table 1). Homogenize culture by gentle pipetting; remove 1 mL and place in 1.5 mL spectrophotometer cuvette.
6. Measure optical density; adjust OD₆₀₀ to 0.4 by adding liquid inoculation medium according to the following formula:

$$\frac{\text{OD}_{600} \text{ value}}{0.4} \times \text{mL remaining culture} = \text{final total volume.}$$

7. Add appropriate volume of 200 mM acetosyringone stock to achieve 100 µM final concentration.
8. Use culture immediately or keep at 4°C for short periods until used for transformation.

3.5. *Agrobacterium* Cocultivation with IEs (see Note 9)

1. Place 1 mL bacterial culture (see Subheading 2.5) in 1.5 mL microfuge tube containing IEs; gently invert tubes and incubate at RT 15 min.
2. Place 25 IEs scutellum side-up on solid cocultivation medium (see Note 10, Table 1).
3. Seal petri plates with parafilm to reduce culture contamination and dessication; incubate in darkness for 2 days at 24°C.
4. Transfer IEs to callus-induction medium (CIM; see Table 1, Note 11) with 100 mg/L carbenicillin for 4 weeks (see Note 12) to inhibit *Agrobacterium* growth before selection. Visible coleoptiles should be removed as soon as observed.
5. After 10 days on CIM, screen IEs for GFP expression (see Note 13, Fig. 2). Keep those IEs expressing GFP on CIM; eliminate IEs with no GFP expression, no callus or those which die during culture.

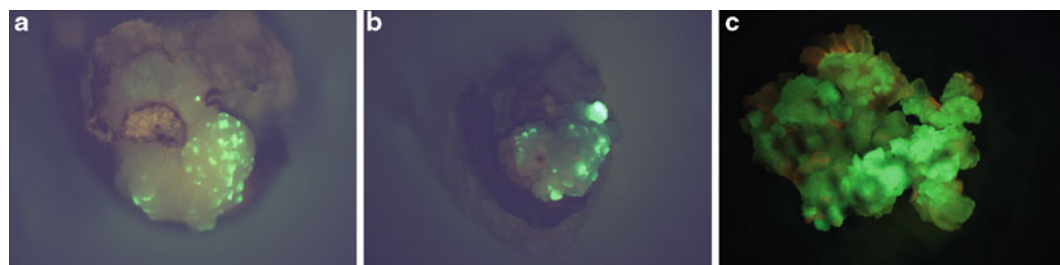


Fig. 2. P898012 immature embryos infected with *Agrobacterium tumefaciens* with the transformation vector, pPZP201-pGFP-PMI, cultured on callus induction medium (see Table 1) for (a) 10 days and on mannose selection medium (see Table 1) for (b) 30 days and (c) 60 days and visualized for GFP fluorescence. Magnification = ×20.

6. During culture on CIM, subculture IEs every other week, or sooner if heavy phenolic production is observed (see Note 14).
7. Continue to transfer and track GFP-expressing calli (see Note 15) on selection medium (see Subheading 3.6).

3.6. Culturing of Tissue, Selection, and Regeneration

Three-stage mannose selection involves successively lowering levels of mannose and sucrose (see Note 16).

1. Culture GFP-expressing calli (see Subheading 4.1) in the dark for 2 weeks on Selection Medium I (see Table 1).
2. Transfer GFP-expressing calli to Selection Medium II (see Table 1) for 2 weeks in the dark.
3. For the final 2 weeks, culture in the dark on Selection Medium III (see Table 1).
4. For slower growing calli, third stage of selection can be extended for two additional weeks.
5. Transfer selected calli to Regeneration Medium (see Table 1); incubate at low-light intensity ($85 \mu\text{mol/s/m}^2$) with 16 h/8 h light/dark for ~4 weeks. For first 5 days of regeneration, cover petri plates with white paper to acclimate calli to light (see Note 17).
6. Transfer ~3–5 cm shoots to Rooting Medium (see Table 1); place at $100 \mu\text{mol/s/m}^2$ with 16 h/8 h light/dark for 4–6 weeks.
7. All regeneration and rooting media contain only 1% mannose plus 100 mg/L carbenicillin.
8. After 4–6 weeks on Rooting Medium, transfer shoots with ≥ 1 long root (see Note 18) to small pots with Supersoil; transfer to larger pots as plants grow.
9. Plants are grown initially in the Conviron E-15 growth chamber with a 16 h/8 h light/dark period under fluorescent and incandescent lighting ($250 \mu\text{mol/s/m}^2$) and day/night temperatures of 25/20°C.
10. When plants are 8–10" tall, transfer to Kolpak walk-in growth chamber under same growth conditions or to greenhouse; water and fertilize as for donor plants (see Subheading 2.1).

4. Analysis

4.1. Visual Screening for GFP Expression

1. Observe IEs using epifluorescence stereomicroscope [Zeiss Lumar V12 fluorescence dissecting scope: filter set (Chroma Endow): excitation filter, BP470-490; emission filter, BP 505–550]

attached to Q Imaging Megapixel camera with iVision 4.0.15 imaging software.

2. Track GFP-expressing calli by circling with a marker on the bottom of the petri plate.

4.2. Genomic DNA and Protein Expression Analyses

1. Although the technical aspects of analyzing genomic DNA and protein expression are not described in this review, confirming stable transformation and independence of transgenic events is necessary to determine accurate stable transformation frequencies.

5. Notes

1. YEP medium contains 10 g/L yeast extract (Becton Dickinson Difco: 212750), 10 g/L Bacto Peptone (Becton Dickinson Difco: 211677), 5 g/L NaCl (Fisher: BP 358–212); adjust pH to 7.2 with 1 N NaOH. Solid medium contains 18 g/L Bacto-agar (Becton Dickinson Difco: 214010). Autoclave for 22 min at 121°C. Store at 4°C.
2. Plants should be grown under optimal conditions as quality of donor plants affects capacity of IEs to produce embryogenic callus. During winter months in colder climates, prewarmed water should be used to water donor plants.
3. Collect spikes from plants ~12–14 days postpollination when IEs are ~1.5 mm; they are not transparent but just turning cloudy due to starch accumulation. Younger IEs, being nearly transparent, are difficult to locate and do not give the appropriate culture response. The developmental stage that is ideal for successful transformation varies among genotypes, but for P898012 the optimal size range is 1.5–2.5 mm.
4. For optimum response, use IEs taken from spikes immediately or within 1 day after collection, as callus response declines with time.
5. Prolonged periods of sterilization may damage IEs; insufficient sterilization may result in microbial contamination.
6. IE excision can be performed in several ways; the most important aspect being to minimize explant damage. To excise IEs locate notched side of the seed as the side opposite the notch is the location of the embryo. Firmly hold the seed with forceps so the notched side faces down; with a scalpel peel back the pericarp and seed coat around the tip. Use the scalpel to carefully lift out the IE without damaging it and place it in liquid inoculation medium.

7. Heating P898012 IEs at varying temperatures for 3 min before *Agrobacterium* infection increases to varying degrees frequencies of GFP-expressing calli, of mannose-selected calli and of transformed calli. The most optimal treatment for this variety is 43°C for 3 min with cooling at 25°C. When this optimal heat pretreatment was done prior to inoculating IEs of three other varieties, Tx430, Tx623, and N247, frequency of calli expressing GFP after 10 days also increased compared to non-heat-treated controls; however, variation in temperature and time of heat treatment were not attempted in order to optimize the response, and stable transformation efforts were not completed. That heat treatment increased transformation frequencies is perhaps due to the fact that short periods of heat shock reverse cell death. For P898012, longer 43°C heat treatments (5, 10, and 30 min) decrease numbers of GFP-expressing IEs and tissues surviving selection.
8. Centrifuging P898012 IEs before inoculation either without heat or with heat at various temperatures decreases frequencies of all tissue responses. Using both heat and centrifugation, however, increases tissue de-differentiation of P898012.
9. Methods used for inoculation and cocultivation of IEs are critical for successful callus induction and all other tissue culture steps. Other varieties in which we have attempted certain aspects of this procedure are as follows: Tx430, an elite variety with robust in vitro performance (26); Tx623, the variety for which the genome was recently sequenced (27); and N247, a short-season variety (28). It is well known that IEs of some sorghum varieties respond more negatively to the stress of *Agrobacterium* inoculation than others (16); this response is manifested by tissue blackening after cocultivation and secretion of phenolics.
10. It is advisable to culture only 25 IEs spread out on each petri plate to encourage callus formation; examine cultures daily.
11. Callus induction from IEs and growth rate of tissues is genotype-dependent.
12. The length of callus induction can be decreased to 1 week, depending on the genotype. When calli have visible white, nodular, embryogenic structures and phenolic secretion subsides, tissues can be transferred to selection medium. Because recovery from cocultivation varies by genotype, resting period lengths vary.
13. Although low-level GFP expression can be observed at 2–3-day postinfection, transient GFP expression is better scored at 10-day postinfection (see Fig. 2a). Visual monitoring of fluorescence can continue during selection and regeneration as expression levels increase over time (see Fig. 2b, c).

GFP expression at 10-day postinoculation is distinct and notable and can be used to compare different treatments. Nearly all GFP-expressing cells develop from the scutellum; this side grows rapidly, forming large masses of callus. During regeneration, most shoots developing from callus that survives selection express GFP.

14. If the sorghum variety produces noticeable amounts of phenolics, 1% PVP can be added to all media to reduce tissue blackening. Despite adding PVP, heavy phenolic production can still occur. Close monitoring of culture materials and transfer, sometimes daily, to fresh medium is necessary, if blackened or dead tissues exist; transfer only nonblackened tissues. Most sorghum varieties produce phenolics immediately following *Agrobacterium* inoculation with the amount increasing during cocultivation, but decreasing after the cocultivation period.
15. To facilitate tracking GFP-expressing tissues, circle tissues on the bottom of the petri plate with a permanent marker. During selection and regeneration steps, calli from a single embryo are tracked by drawing sectors around the tissues on the bottom of the petri plate or etching a furrow in the agar around the tissue.
16. For optimal mannose selection, sucrose is used in combination with mannose because it is believed that sucrose significantly decreases the phytotoxicity of mannose. Nontransgenic cells turn dark brown and then black, failing to survive; putative transgenic cells do not discolor, and maintain an opaque, white color and continue to express GFP.
17. Regenerability of transformed calli can vary between varieties owing to the fact that many cultivars lose the ability to re-differentiate into plants after many weeks in culture.
18. For shoots with at least one long root, gently loosen agar with forceps, pull shoots from agar slowly, rinse with luke-warm tap water to remove excess agar and plant in soil.

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Chapter 11

Split-Transgene Expression in Wheat

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Abstract

The establishment of traits that result from the concerted expression of complementing transgene fragments is a feasible tool for trait control or gene flow control in plants. This chapter describes the methodology for producing herbicide-resistant and pollen-sterile wheat plants by the intein-mediated assembly of inactive precursor protein fragments (protein *trans*-splicing). We suggest the design of intein-containing vectors for split-transgene expression. We describe transient plant assays that can be used to analyse the functionality of the system and describe the transformation of wheat plants using a split selection marker.

We hope that this chapter will be a helpful guideline for researchers who are interested in applying similar split-gene approaches in wheat or other monocotyledonous crops.

Key words: Inteins, Protein *trans*-splicing, *Triticum aestivum*, Barnase, Male sterility, Acetolactate synthase

1. Introduction

Commonly, the establishment of transgenic plant traits relies on the expression of DNA sequences that encode an active protein. Alternatively, the traits can be established by engineering functional proteins through the assembly of inactive precursor peptides that are expressed from independent expression cassettes. Strategically, such “modular” production of recombinant proteins enables an operator to switch on the production of active proteins (or the establishment of a trait) by combining the complementary expression units in one cell. For example, this combination can be achieved by crossing plants (hybridization). Conversely, the production of an active recombinant protein can be inhibited, provided that physical separation of the gene fragments can be achieved (e.g., by chromosomal segregation). Therefore, in the future, split-gene systems

might play a role in the approaches concerning trait control or gene containment or in the technologies for plant hybrid breeding (1, 2).

The initial split barnase system in plants was based on the non-covalent association of barnase domains in a tomato, which assemble into a cytotoxic protein (3). Although it was functional, the technology suffered from the instability of the non-covalently associated barnase complex at higher temperatures. Thus, in order to create systems that are more suitable for open-field conditions, inteins can be used to covalently link the protein domains (precursor proteins). Inteins are sometimes referred to as “protein introns.” They disrupt the coding sequence of a gene (the “extein” coding sequences). However, in contrast to introns, inteins are translated together with their host protein and excised consecutively by a mechanism termed protein splicing (4). The reaction includes the covalent linkage of the adjacent host protein sequences, thus making inteins a suitable tool for the *in vivo* assembly of protein domains in *trans*. In plants, intein-mediated protein splicing was used to assemble recombinant proteins in tobacco (5, 6), *Arabidopsis* (7, 8), and wheat (9).

This chapter provides protocols for the production of wheat plants that are male-sterile as a result of expressing the domains of a toxic ribonuclease (barnase) in a tissue that is important for pollen development (tapetum) and fusing them using the *Synechocystis* sp. *DnaB* intein system. Furthermore, we describe how a mutated acetolactate synthase (ALS) protein from rice can be used for the herbicide selection of transgenic wheat when it is assembled from precursor molecules by the *Synechocystis* sp. *DnaE* intein system. We provide a protocol for how intein systems can be analysed by rapid transient plant assays.

2. Materials

2.1. Cloning Procedures

1. Proofreading polymerase with appropriate buffer and dNTP mixture.
2. Standard purification kit for PCR products.
3. T4 DNA Ligase.
4. Competent *Escherichia coli* cells (e.g., XL-1 Blue, DH5 α).
5. LB plates containing the appropriate antibiotics.
6. Binary vector.
7. (Optional) Standard blunt-end cloning kit.

2.2. Plant Material

1. Eight-week-old *Nicotiana benthamiana* plants grown under greenhouse conditions with 16 h of light at 20°C and 8 h of darkness at 18°C (60% relative humidity, circulating air).

2. 2–4-week-old wheat callus culture (production described in Subheading 3.3, item 1).
3. Spring wheat (*Triticum aestivum* L., cultivar “Bobwhite”) grown under greenhouse conditions with 16 h of light at 20°C and 8 h of darkness at 16°C (60% relative humidity, circulating air).

2.3. Transient Assays Using Agroinfiltration

1. *Agrobacterium tumefaciens* strain GV3101::pMP90 (10).
2. LB medium containing 100 mg/L rifampicin, 25 mg/L gentamicin, and an antibiotic agent for selecting the binary vector (depends on the selection marker that is used; in case of kanamycin or carbenicillin, a concentration of 50 mg/L was used).
3. Infiltration buffer consisting of 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 10 mM magnesium sulphate, pH 5.5.
4. GUS staining solution consisting of 50 mM sodium phosphate buffer, pH 7.0; 1 mM EDTA, pH 8.0; 0.1% (v/v) Triton-X 100; 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc).

2.4. Transient Assays Using Particle Bombardment

1. Prepare a 0.8 mM copper (II) sulphate pentahydrate stock solution and store at 4°C.
2. Prepare a 2,4-D stock solution by diluting 2.36 g/L of (2,4-Dichlorophenoxy)acetic acid sodium salt monohydrate (Sigma D-6679) in distilled water and store at 4°C.
3. Pre-transformation medium (MS culture medium (11) containing MS inorganic salts including vitamins (Duchefa, M0222), 100 g/L sucrose, 1 g/L N-Z-Amine A, 1 mL/L copper (II) sulphate pentahydrate stock solution, 2 mL/L 2,4-D stock solution, and 5.2 g/L Phytigel, pH 5.8–5.9 (see Note 1)).
4. For preparation of the Micron gold suspension (Microcarriers), sterilise 60 mg of gold particles (0.6 μ m gold; BIO-RAD) with 1 mL of 70% ethanol in a microcentrifuge tube by shaking briefly and incubating for 15 min. Sediment the particles by centrifugation at 13,000 $\times g$ for 5 min, carefully remove supernatant, and add 1 mL of sterile water. Centrifuge the tubes and discard the supernatant as before. Repeat the last washing step twice. After the final washing step, resuspend the particles in 1 mL of 50% glycerol.
5. For particle coating, quickly mix 50 μ L of the Micron gold suspension, 10 μ g DNA (maximal volume 10 μ L), 50 μ L of 2.5 M calcium chloride, and 20 μ L of 0.1 M spermidine; shake for 2 min; incubate at room temperature for 10 min; centrifuge at 13,000 $\times g$ for 20 s; wash once with 70% ethanol and once with 99.5% ethanol; and resuspend in 60 μ L of 99.5% ethanol.

6. For co-transfection, mix the plasmids at a 1:1 ratio (5 µg each).
7. Macrocarriers, stopping screens, and 900- and 1,100-psi rupture discs (BIO-RAD).
8. Biolistic PDS-1000/He Particle Delivery System (BIO-RAD).
9. GUS staining solution described in Subheading 2.3.

2.5. Transformation of Wheat Plants

1. Material for particle bombardment is found in Subheading 2.4. Use the 900-psi rupture discs.
2. MS culture medium (11) containing MS inorganic salts including vitamins (Duchefa, M0222), 30 g/L sucrose, 1 g/L N-Z-Amine A, 1 mL/L copper (II) sulphate pentahydrate stock solution, 2 mL/L 2,4-D stock solution, and 5.2 g/L Phytigel, pH 5.8–5.9 (see Note 1).
3. Pre-transformation medium is the MS culture medium containing 100 g/L sucrose.
4. Post-transformation medium is the MS culture medium containing 60 g/L sucrose.
5. Prepare a 1% ethylenediaminetetraacetic acid, ferric sodium salt (FeNa-EDTA) solution and store at 4°C.
6. Prepare a 1-mM primisulphuron methyl (PSM) stock solution in DMSO, filter sterilise, and store at –20°C.
7. Prepare a 10-mM imazethapyr (IMA) stock solution in DMSO, filter sterilise, and store at –20°C.
8. Callus selection medium contains MS inorganic salts including vitamins (Duchefa, M0222), 30 g/L sucrose, 1 g/L N-Z-Amine A, 1 mL/L copper (II) sulphate pentahydrate stock solution, 1 g/L 2-(N-morpholino)ethanesulphonic acid (MES), 7 mL/L FeNa-EDTA solution, 2 mL/L 2,4-D stock solution, and 5.2 g/L Phytigel, pH 6.0. After autoclaving, add 1 mL/L of the PSM stock solution and 0.5 mL/L of the IMA stock solution (see Note 1).
9. For preparation of the kinetin solution, dilute to 10 g/L in DMSO and store at –20°C.
10. For preparation of the zeatin riboside solution, dilute to 100 g/L in DMSO and store at –20°C.
11. MS regeneration medium contains MS inorganic salts including vitamins, 20 g/L maltose, 1 g/L N-Z-Amine A, 1 mL/L copper (II) sulphate pentahydrate stock solution, 100 µL/L kinetin solution, and 5.2 g/L Phytigel, pH 5.8–5.9. After autoclaving, add 70 µL/L of the kinetin solution.
12. For preparation of the B1-*myo*-inositol solution, dilute 1 g/L of thiamine HCl and 100 g/L of *myo*-inositol in distilled water and store at –20°C.

13. Plant selection medium contains half-strength MS inorganic salts including vitamins (Duchefa, M0222), 15 g/L sucrose, 0.1 g/L N-Z-Amine A, 1 mL/L copper (II) sulphate pentahydrate stock solution, 0.5 mL/L B1-*myo*-inositol solution, and 5.2 g/L Phytigel, pH 5.8–5.9. After autoclaving, add the 0.5 mL/L PSM stock solution (see Note 1).

2.6. Analysis of Pollen Fertility

1. Prepare the following solutions: 1% (w/v) Malachite Green in 95% ethanol, 1% (w/v) Acid fuchsin in distilled water and 1% Orange G in distilled water.
2. Prepare the pollen-staining solution (12) by adding the components in the following order: 10 mL of 95% ethanol, 1 mL of Malachite Green solution, 50 mL of distilled water, 25 mL of glycerol, 5 mL of phenol (equilibrated), 5 g of chloral hydrate, 5 mL of Acid fuchsin solution, 0.5 mL of Orange G solution, and 2 mL of glacial acetic acid (see Note 2). Shake after the addition of each ingredient.

3. Methods

This section has been subdivided into four parts: (1) design of vectors for split-gene expression, (2) test of intein-mediated protein assembly in plants by transient assays, (3) transformation of wheat plants, and (4) analysis of plant traits resulting from split-gene expression.

The experiments include the intein-mediated assembly of β -glucuronidase (GUS), of mutated rice acetolactate synthase (ALS, which confers resistance to herbicides such as PSM and IMA), and of a cytotoxic RNase from *Bacillus amyloliquefaciens* (Barnase) that leads to male sterility when expressed in the tapetum (tissue that is necessary for pollen development).

3.1. Design of Split-Gene Expression Vectors

The chemical nature of the splice site junction determines the efficiency of the splicing reaction. We suggest complying with the following rules:

Rule 1: The first step in designing a split-gene expression system is to choose an appropriate position for the splicing junction within the protein of interest. It is strongly recommended not to dissect the gene within important regions or functional domains. These areas can be identified through a sequence alignment of homologous genes from different organisms under the assumption that structures of high similarity are conserved and putatively important for gene function.

Rule 2: Most inteins contain three highly conserved splice junction residues, which include a Cys or Ser at the beginning of the intein and a His-Asn at the end of the intein. An additional key amino

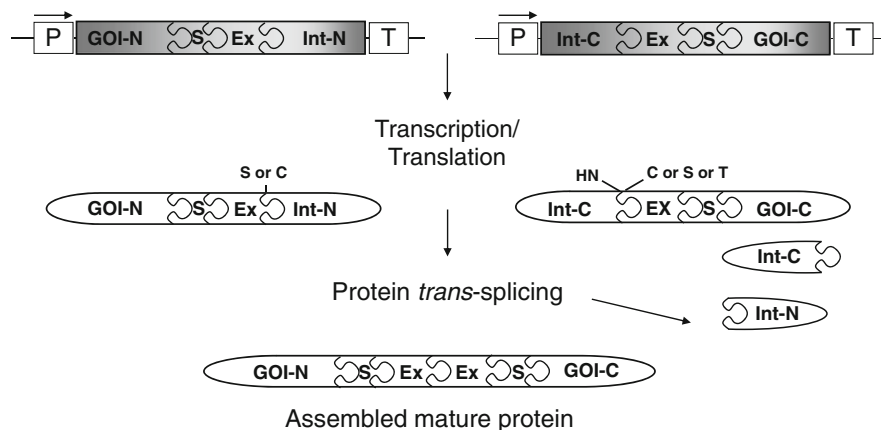


Fig. 1. General design of the expression vectors containing split inteins (not drawn to scale). The N- and C-terminal parts of the system can be located on the two vectors (as has been used in the transient assays described in the text) or on one T-DNA (as been used in wheat transformation experiments). The positions of key *trans*-splicing residues are indicated. *GOI-N* N-terminal fragment from the gene of interest, *GOI-C* C-terminal fragment from the gene of interest, *S* spacer consisting of 1–3 flexible GGGGS linker sequences, *Ex* extein part of the intein host protein (4–7 amino acids), *T* terminator, *P* promoter (depending on the objective of the experiment, constitutive or tissue-specific promoters can be used).

acid for *trans*-splicing is a Ser, Thr, or Cys as the first amino acid of the C-extein (13). The presence of these amino acids is essential for the protein *trans*-splicing mechanism (as has been demonstrated by site-specific mutagenesis (9), see Note 3).

Rule 3: It is assumed that the introduction of short extein stretches derived from the intein host protein at the splice junction site are beneficial for the efficiency of the *trans*-splicing reaction (14).

Rule 4: The insertion of flexible GGGGS linker sequences, which space the fusion domains of the assembled barnase protein, significantly increases the system's efficiency in plants (9) and may also be favourable in other intein-mediated split-gene systems.

For the assembly of ALS, we used the N- and C-terminal intein sequences from the *DnaE* gene of *Synechocystis* sp. For assembly of the barnase and GUS proteins, we used the split intein from the *DnaB* gene of *Synechocystis* sp. The N-terminal extein sequences are RESG for the *DnaB* intein and DVKFAEY for the *DnaE* intein. The C-terminal extein sequences are SIEQD for the *DnaB* intein and CFNGH for the *DnaE* intein. Each spacer sequence consists of 1–3 flexible GGGGS linkers.

For detailed sequence information, see refs. 7, 9.

Figure 1 summarises the general design of the split intein containing the expression vectors.

3.2. Analysis of Split-Transgene Expression Using Transient Plant Assays

This section describes two possible experimental strategies for rapid testing of the intein-mediated split-gene system, agroinfiltration, and particle bombardment of the plant tissue. The assays are helpful for streamlining the experimental pipeline, since they

deliver valuable information for the vector design prior to the stable transformation of wheat.

3.2.1. Delivery of the Split GUS System and the Split Barnase System by Agroinfiltration of N. benthamiana Leaves

1. Transform the *A. tumefaciens* strain GV3101::pMP90 with the split-gene expression vectors.
2. Grow a 20-mL overnight culture of *Agrobacterium* solution in LB medium with the appropriate antibiotics.
3. Harvest the cells by centrifugation for 15 min at $4,500 \times g$ and 4°C .
4. Resuspend the cells in infiltration buffer (see Note 4).
5. Determine the optical density (OD) at 600 nm and dilute to an OD of 0.5.
6. Mix the *Agrobacteria* cultures containing the N- and C-terminal gene fragments at a 1:1 ratio.
7. Agroinfiltration was conducted on fully expanded leaves still attached to the intact plant. Slightly scratch the lower side of a leaf from an 8-week-old *N. benthamiana* plant with a pipette tip or a blade, and infiltrate the *Agrobacteria* solution using a syringe without a needle (see Note 5).

3.2.2. Delivery of the Split GUS System by Particle Bombardment of the N. benthamiana Leaves and Wheat Callus

1. Cut 5-cm² leaf pieces of *N. benthamiana*. Transfer leaf material or wheat callus to the centre of a Petri dish with pre-transformation medium (see Note 6). Incubate for 6 h at 22°C in the dark.
2. Use the following settings: 1-cm distance between the rupture disc and macrocarrier, 4-cm distance between the stopping screen and target tissue, 28 mmHg.
3. Sterilise the chamber and all components with 70% ethanol.
4. Sterilise the macrocarrier holders, macrocarriers, stopping screens, and rupture discs by dipping them in absolute ethanol and allow it to evaporate completely.
5. Briefly vortex the coated gold particles, place 6 μL onto the macrocarrier membrane, and allow for complete drying.
6. Load a rupture disc (1,100 psi for *N. benthamiana* leafs and 900 psi for wheat callus) into the rupture disc retaining cap and firmly tighten the screw into place.
7. Place a stopping screen into the microcarrier launch assembly.
8. Invert and position the macrocarrier holder containing the macrocarrier and coated gold particles over the stopping screen.
9. Place a sample on the target stage, draw a vacuum, and fire the gun (see Note 7).

3.2.3. *Histochemical Analysis of GUS Activity in the Infiltrated Leaves of N. benthamiana or Bombarded Wheat Callus*

1. Infiltrated or bombarded *N. benthamiana* leaves and bombarded wheat calli are stained using GUS staining solution 2–3 days after transformation.
2. Transformed tissue is infiltrated with an appropriate amount of GUS staining solution twice for 10 min under vacuum (see Note 8).
3. Samples are incubated for 4 h at 37°C in the case of leaf material and for 24 h at 37°C in the case of callus tissue.
4. Remove the staining solution, add 70% ethanol for destaining, and shake slightly overnight.

Figure 2 depicts the GUS expression that results from the delivery of split transgenes into plant tissue.

3.2.4. *Analysis of Barnase Activity in the Infiltrated Leafs of N. benthamiana*

Record the degree of necrosis in the infiltrated *N. benthamiana* leaf material 3, 6, and 9 days after infiltration. An example of the results of an infiltration experiment is given in Fig. 3.

3.3. *Transformation of Wheat Plants*

This subchapter describes the delivery of split barnase and split ALS constructs into spring wheat plants using the particle bombardment technique.

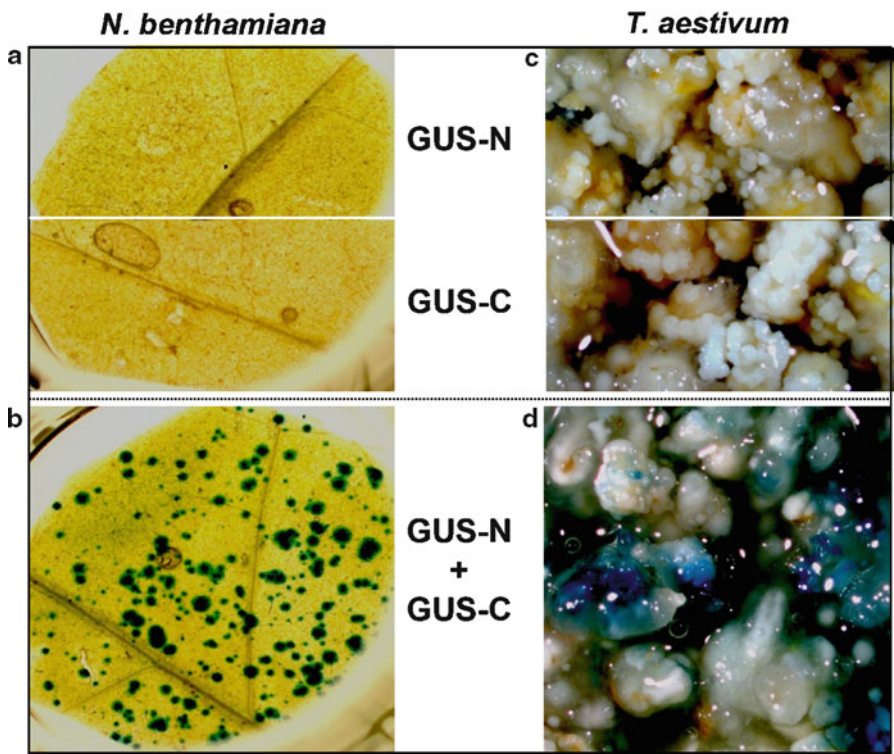


Fig. 2. Split GUS expression in bombarded *Nicotiana benthamiana* leaves (a, b) and wheat callus tissue (c, d).

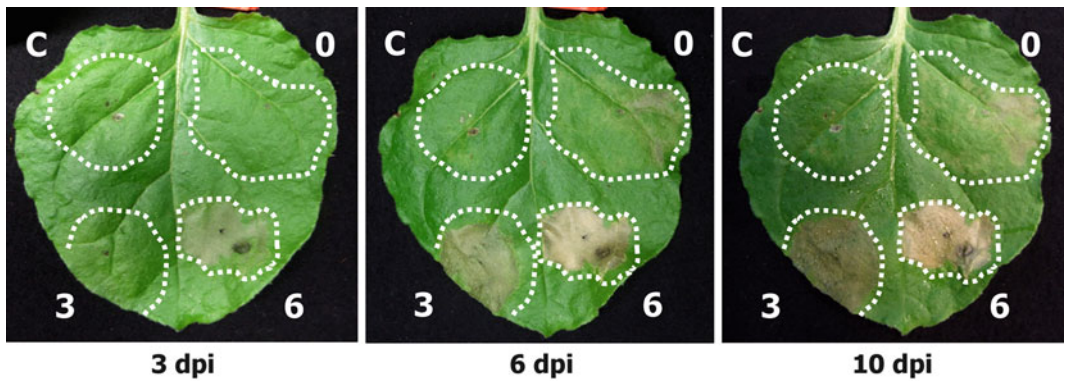


Fig. 3. Transient assay monitoring activity of the assembled barnase. Leaves were infiltrated with different mixtures of *Agrobacteria* carrying T-DNAs for constitutive N- and C-terminal *barnase* expression. The degree of necrosis is an indicator of system efficiency. The numbers indicate the total number of GGGGS linkers between the N- and C-terminal fusion domains of the mature barnase protein; C is the negative control (infiltration of an *Agrobacterium* mix carrying N-terminal expression vector only). dpi days post infiltration. Note the positive effect of spacer length on the formation of active barnase.

3.3.1. Isolation of Wheat Embryos and Callus Culture Maintenance

1. Cut spikes of wheat plants 14–18 days after anthesis (8–10-week-old plants) and carefully remove the immature caryopses by hand (see Note 9).
2. Perform a surface sterilisation by the successive immersion of seeds in 70% ethanol for 1–2 min and freshly prepared 2.5% sodium hypochlorite/0.01% SDS solution for 15 min.
3. Wash the seeds three times in sterile, distilled water.
4. Aseptically excise the immature embryos (1.0–1.5 mm in length, semitransparent) with forceps and a scalpel using a stereomicroscope and place them, scutellum side up, on MS culture medium (see Note 10).
5. Allow the embryogenic callus to develop in the dark at 25°C for 3–4 weeks.
6. Select the embryos that develop compact nodular calli using a stereomicroscope.

Examples of an immature embryo and an embryogenic callus of wheat are given in Fig. 4.

3.3.2. Ballistic Transformation of Wheat Plants

The technical protocol for micro-projectile bombardment of embryogenic callus is analogous to the ballistic bombardment of *N. benthamiana* leaves and wheat callus (see Subheading 3.2):

1. Pretreat the calli for 6 h on pre-transformation medium at 22°C in the dark.
2. For each bombardment, place approximately 50 callus cultures in the middle of a plate in a ring-like formation (radius of about 1 cm). In that position, the probability of hits is high.

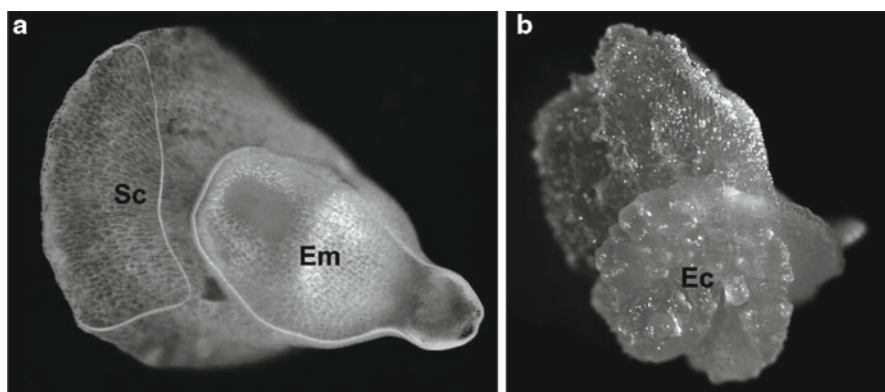


Fig. 4. Immature embryo (a) and embryogenic callus (b) of wheat. *Sc* scutellum, *Em* embryo, *Ec* embryogenic callus.

3. Perform the particle bombardment using 900-psi rupture discs according to Subheading 3.2.2.
4. Transfer the bombarded cultures to 22°C in the dark overnight.
5. Incubate the cultures on post-transformation medium for 2 weeks at 25°C in the dark.

3.3.3. Callus Culture Selection for Split ALS

1. Transport the bombarded calli to callus selection medium (containing IMA and PSM) for about 2 months at 22°C in the dark. Change medium two to three times (see Note 11). Transfer only white calli to the fresh medium.
2. Transfer the selected calli to MS regeneration medium for about 4–6 weeks with 16 h of light at 24°C and 8 h of dark at 15°C (3000 Lux). Change the medium every 2–3 weeks (see Note 11).
3. Move the regenerating plantlets to jars containing plant selection medium under light conditions for 4 weeks.
4. Acclimatise the fully developed plantlets for 7–14 days in liquid medium containing a fourfold dilution of MS salts or dip the roots of the plantlets in a mixture of 0.4% (w/w) indole-3-butyric acid (IBA) in talcum.
5. Transfer the plants into soil and grow under greenhouse conditions to maturity.

3.4. Analysis of the Phenotypes Resulting from Split-Gene Expression

3.4.1. Male Sterility

Male sterility of wheat plants can be determined by pollen staining (12) or by visual inspection of mature ears. Pollen staining allows for the early identification of sterile flowers that can be used as pollen acceptors:

1. Isolate the anthers from two to three different florets of one ear 2 days before anthesis (yellowish green colour) and transfer them to glass Petri dishes (see Note 12).

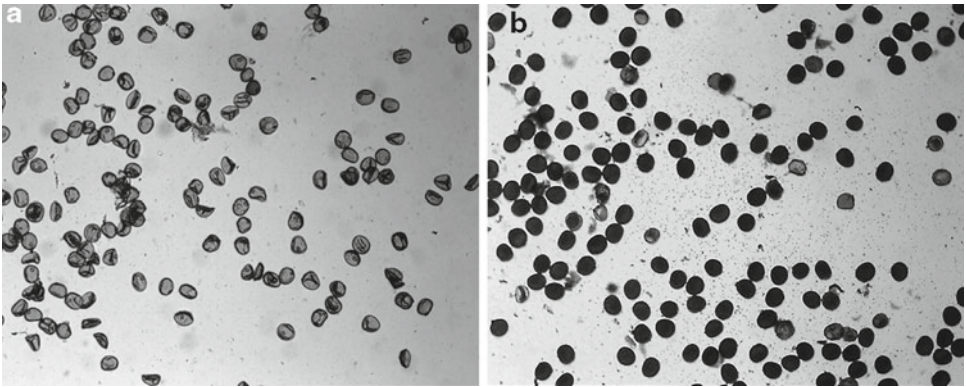


Fig. 5. Morphology of the wheat pollen produced by a plant expressing a split *barnase* transgene under the control of a tapetum-specific promoter (a) and a non-transgenic plant (b) under identical magnification.

2. Add a few drops of staining solution.
3. Tease apart the anthers with two fine forceps.
4. Observe the colour of the pollen 5–15 min later under a light microscope that can optionally be equipped with differential interference contrast (DIC).
5. Fertile pollen grains contain a cytoplasm that appears red-purple and has a globular shape. In contrast, sterile pollen appears transparent and deformed (Fig. 5).

3.4.2. Herbicide Tolerance of Plants Grown from Seed

The herbicide resistance of plants can be assayed by selection on half-strength MS medium containing 0.5 μ M PSM using the following protocol (see Note 13):

1. Perform a surface sterilisation according to Subheading 3.3.1.
2. Let the seeds germinate in Petri dishes on plant selection medium without PSM for 3–7 days.
3. Transfer the plantlets to jars with plant selection medium containing PSM.
4. Select the surviving plants and follow the steps outlined in Subheading 3.3.3.

4. Notes

1. Autoclave all media for 15 min at 121°C. Use Phytigel directly after autoclaving and cooling the solutions to 45°C.
2. Prepare the pollen-staining solution in the fume hood. Chloral hydrate is a strong sedative and harmful. Dispose of the remaining solution in the appropriate waste container.

3. A comprehensive list of intein sequences is given by New England Biolabs (<http://www.neb.com/neb/inteins.html>.) and includes useful information about the biochemistry and structure of inteins.
4. Resuspend by carefully pipetting and gentle shaking.
5. Water the *N. benthamiana* plants 15 min before infiltration. Use full-grown leaves, but not the oldest. Scratch the lower side of the leaf, producing an incision of about 1 mm. Use a 5-mL syringe without a needle. The use of a face mask is strongly recommended during the infiltration procedure.
6. The step includes an osmotic pretreatment.
7. Follow the manufacturer's instructions (according to http://www.bio-rad.com/LifeScience/pdf/Bulletin_9075.pdf).
8. For efficient infiltration of the buffer, cut small pieces of leaf material (about 1 cm²) and transfer to small jars before adding the staining solution.
9. Be careful not to injure the caryopsis before surface sterilisation.
10. To excise the embryo, hold the caryopsis with the embryo side on the top. Cut just beneath the embryo, remove the seed coat, and transfer the embryo to MS culture medium using the tip of the scalpel. Be careful not to injure the embryo or scutellum.
11. How often the medium needs to be changed depends on the amount of degradation compounds produced and dispensed through the medium. Their presence can be recognised by a dark colouration of the medium.
12. For the isolation of anthers, use fine and exact tweezers. In order to prevent drying of the anthers, cover the Petri dishes after isolation and quickly add staining solution.
13. Always use a non-transformed plant as a control on selective medium.

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Chapter 12

Agrobacterium*-Mediated Transformation of *Brachypodium distachyon

Vera Thole and Philippe Vain

Abstract

Brachypodium distachyon is an attractive genomics and biological model system for grass research. Recently, the complete annotated genome sequence of the diploid line Bd21 has been released. Genetic transformation technologies are critical for the discovery and validation of gene function in *Brachypodium*. Here, we describe an efficient procedure enabling the *Agrobacterium*-mediated transformation of a range of diploid and polyploid genotypes of *Brachypodium*. The procedure relies on the transformation of compact embryogenic calli derived from immature embryos using either chemical selection alone or a combination of chemical and visual screening of transformed tissues and plants. Transformation efficiencies of around 20% can routinely be achieved using this protocol. In the context of the BrachyTAG programme (BrachyTAG.org), this procedure made possible the mass production of Bd21T-DNA mutant plant lines.

Key words: *Brachypodium*, Transformation, Transgenic plants, Bd21, Green fluorescent protein, T-DNA tagging, Functional genomics, BrachyTAG

1. Introduction

In recent years, *Brachypodium distachyon* (hereafter *Brachypodium*) has emerged as a genomics and experimental model system for grass research. *Brachypodium* plant and genome attributes made possible the investigation of biological questions relevant to large and genetically complex cereal crops (e.g. wheat, barley) and biomass grasses (e.g. elephant grass or switchgrass). *Brachypodium* is a small, easy-to-cultivate, self-fertile plant with a short life cycle (less than 4 months) and a small genome size (approximately 272 Mb harbouring 25,532 protein-coding loci). In 2010, the annotated genome sequence of the diploid line Bd21 was published (1)

providing a foundation sequence for the *pooid* grasses. At the same time, efficient transformation technologies have also been developed for *Brachypodium* (2–5).

Efficient genetic transformation systems are essential to exploit the information from genomic sequences and to facilitate gene discovery and functional genomics programmes. In the past, biolistic (2, 3) and *Agrobacterium*-mediated transformation (4, 5) systems have been developed for a range of *Brachypodium* genotypes (see ref. 6 for review and references within). Embryogenic calli produced from immature explants (seeds or embryos) are generally used as the target for gene delivery. Transformation efficiencies above 20% are routinely obtained via *Agrobacterium*-mediated transformation. Here, we describe an efficient transformation system which has been used for the transformation of diploid and polyploid *Brachypodium* genotypes (Table 1). The procedure can be implemented using chemical selection alone (i.e. hygromycin) which is the preferred approach for the over-expression of genes of interest or RNAi studies. The procedure can also be carried out using both chemical selection (hygromycin) and visual screening (green fluorescent protein, GFP) for high-throughput transformation or technology optimisation (Fig. 1). In the past, this dual selection system made possible the production of a collection of 5,000 fertile Bd21T-DNA plant lines (7) in the context of the BrachyTAG programme (BrachyTAG.org). Future evolution of *Brachypodium* transformation technologies includes the use of mature (instead of immature) explants for embryogenic callus production as well as in planta transformation.

Table 1
Transformation efficiency of diploid and polyploid *Brachypodium* genotypes

Genotype	Origin	Ploidy level ^a	Transformation efficiency ^b (%)
Bd21 (or PI 254867)	Iraq	2n = 10	20
PI 220567	Afghanistan	2n = 30	12.2
TBd 8	Turkey	2n = 20	12
ISK-P2	Turkey	2n = 20 (TBC)	20.1
Bd12-1 (or PI 227011)	Iran	2n = 30	6.2

TBC to be confirmed
^aPolyploid genotypes of *Brachypodium* have been recently characterised as different species: *B. distachyon* (2n=10), *B. Stacei* (2n=20) and *B. hybridum* (2n=30)
^bPercentage of inoculated embryogenic calli producing at least one transgenic plant fully developed in CER

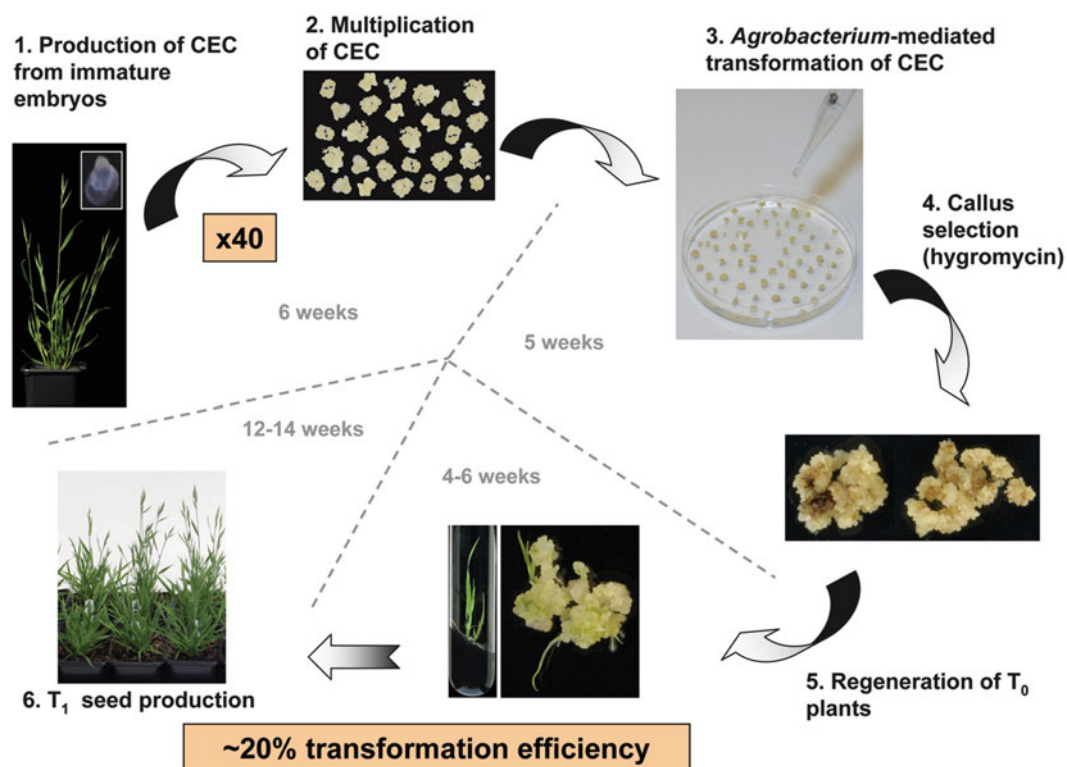


Fig. 1. Flowchart of *Brachypodium* transformation. CEC compact embryogenic callus. $\times 40$ indicates that, on average, 40 CECs are obtained per immature embryo after 6 weeks. Transformation efficiency is the percentage of inoculated CECs producing at least one transgenic plant fully developed in CER.

2. Materials

2.1. Plant Culture

1. Seeds of *B. distachyon* diploid community standard inbred line Bd21 (5, 8) and polyploid genotypes PI 220567, TBd 8, ISK-P2, and Bd12-1 (4, 9; Table 1).
2. Compost mixture for *Brachypodium* whole plant culture: Combine 50% John Innes compost no. 2 with 50% peat and grit mix (870 L Shamrock fine peat, 200 L grit, 2.7 kg Osmocote fertiliser, 1.8 kg sieved garden lime, and 1.8 kg sieved Dolodust).
3. Sodium hypochlorite solution: To prepare 1.3% solution, mix 10 mL sodium hypochlorite (13%, Fluka) with 90 mL deionised H_2O and add one drop of Tween 20. Make fresh as required.

2.2. Bacterial Culture

1. Acetosyringone (AS) stock solution: Prepare 30 mg/mL stock solution by dissolving 600 mg 3',5'-dimethoxy-4'-hydroxyacetophenone (Sigma-Aldrich) in 20 mL dimethyl sulfoxide. Store in 1-mL aliquots at $-20^\circ C$ in the dark (see Note 1).

2. Streptomycin sulphate stock solution: Prepare 100 mg/mL stock solution by dissolving 1 g streptomycin sulphate (Duchefa) in 10 mL sterile deionised H₂O and filter-sterilise. Store in 1-mL aliquots at -20°C.
3. Biotin stock solution: Prepare 1 mg/mL stock solution by dissolving 10 mg biotin (Sigma) in 200–300 µL 1 M KOH and subsequently in a final volume of 10 mL deionised H₂O. Store filter-sterilised solution in 15-mL aliquots at 4°C in the dark.
4. Benedict's test solution: Dissolve 86.5 g C₆H₅Na₃O₇·2H₂O and 50 g Na₂CO₃ in 400 mL deionised H₂O. Dissolve 17.4 g CuSO₄·5H₂O in 50 mL deionised H₂O. Combine both solutions while stirring and dilute to a final volume of 500 mL with deionised H₂O. Store at room temperature in the dark.
5. Solid medium for Benedict's test (LYM): Dissolve 10 g lactose and 1 g yeast extract in a final volume of 1 L deionised H₂O; add 15 g agar no. 1 and autoclave. Aliquot medium into Petri dishes under sterile conditions.
6. Liquid medium for *Agrobacterium* culture (LB + S50): Dissolve 5 g yeast extract, 10 g NaCl, and 10 g peptone from casein in a final volume of 1 L deionised H₂O and adjust pH to 7.2. Autoclave in aliquots of 100 mL, store at room temperature, and before use, add 50 µL streptomycin (100 mg/mL stock) to each 100-mL aliquot (see Note 2).
7. Solid medium for *Agrobacterium* culture (LB + S50 + R50): Dissolve 5 g yeast extract, 10 g NaCl, and 10 g peptone from casein in a final volume of 1 L deionised H₂O. Add 10 g micro agar and adjust pH to 7.2. Autoclave in 100-mL aliquots and store at room temperature. Melt in a microwave oven before use and add 50 µL streptomycin (100 mg/mL stock) and 500 µL rifampicin (10 mg/mL stock) to each 100-mL aliquot. Under sterile conditions, pour approximately 25 mL medium into Petri dishes. Store at 4°C in the dark for up to 2 weeks (see Note 2).
8. Solid medium for *Agrobacterium* culture (MGL + S50 + AS30): Dissolve 5 g tryptone, 2.5 g yeast extract, 5.2 g NaCl, 10 g mannitol, 2.32 g L-glutamic acid sodium salt, 0.5 g KH₂PO₄, and 0.2 g MgSO₄·7H₂O in a final volume of 1 L deionised H₂O. Add 10 g micro agar, adjust pH to 5.5, and autoclave. When the medium has cooled down, add 2 µL biotin (1 mg/mL stock), 500 µL streptomycin (100 mg/mL stock), and 1 mL AS (30 mg/mL stock). Under sterile conditions, pour approximately 25 mL medium into Petri dishes and leave for a few hours to solidify. Store at 4°C in the dark for up to 2 weeks (see Note 2).
9. Liquid medium for *Agrobacterium* suspension and co-culture (MSC + AS45): Dissolve 100 mL 10× macro MS salt solution

(see Subheading 2.3), 10 mL 100× micro MS salt solution (see Subheading 2.3), 10 mL 100× Fe-EDTA solution (see Subheading 2.3), 30 g sucrose, and 30 g glucose in a final volume of 1 L deionised H₂O. Adjust pH to 5.5, autoclave in aliquots of 200 mL, and store at 4°C for up to 3 months. Before use, add 300 µL AS (30 mg/mL stock) to each 200-mL aliquot.

10. Binary vectors: Choose a binary vector suitable for transformation of a grass species. The vector pVec8-GFP (10) containing the hygromycin phosphotransferase (*HPT*) and green fluorescent protein (*sGFPs65T*) genes has been used here as an example. Alternative vectors such as pCLEAN (11) or pBrachyTAG dual binary vectors have also been successfully used to transform *Brachypodium* with this procedure (see Note 2).
11. *Agrobacterium tumefaciens* glycerol stock: Prepare glycerol stock from a single colony of *Agrobacterium* strain AGL1 carrying a binary vector (e.g. pVec8-GFP) grown on LB + S50 + R50 solid medium. Store at -80°C (see Note 3).

2.3. Plant Tissue Culture

1. CuSO₄ stock solutions: Prepare a 250 mg/L stock solution by dissolving 12.5 mg CuSO₄·5H₂O in a final volume of 50 mL deionised H₂O. Prepare a 1 mg/mL stock solution by dissolving 50 mg CuSO₄·5H₂O in a final volume of 50 mL deionised H₂O. Store at 4°C in the dark (see Note 1).
2. CoCl₂ stock solution: Prepare a 280 mg/L stock solution by dissolving 14 mg of CoCl₂·6H₂O in a final volume of 50 mL deionised H₂O. Store at 4°C in the dark.
3. Macro MS salt stock solution: Prepare 10× stock with 19 g KNO₃, 16.5 g NH₄NO₃, 4.4 g CaCl₂·2H₂O, 3.7 g MgSO₄·7H₂O (if anhydrous, use 1.8 g), and 1.7 g KH₂PO₄ in a final volume of 1 L deionised H₂O. Store at 4°C in the dark.
4. Micro MS salt stock solution: Prepare 100× stock with 0.62 g H₃BO₃, 2.23 g MnSO₄·4H₂O, 0.86 g ZnSO₄·7H₂O, 0.083 g KI, 0.025 g Na₂MoO₄·2H₂O, 10 mL CuSO₄ stock solution (250 mg/L), and 9 mL CoCl₂ stock solution (280 mg/L) in a final volume of 1 L deionised H₂O. Store at 4°C in the dark.
5. M5 vitamin stock mixture: Prepare 100× stock with 0.04 g nicotinic acid, 0.05 g thiamine-HCl, 4 g cysteine, 0.2 g glycine, and 0.04 g pyridoxine-HCl in a final volume of 1 L deionised H₂O; adjust pH to 5.8 by adding KOH; and filter-sterilise. Store in aliquots of 50 mL at -20°C in the dark.
6. B5 vitamin mixture: Prepare 100× stock with 100 mg nicotinic acid, 1 g thiamine-HCl, 100 mg pyridoxine-HCl, and 10 g myo-inositol in 1 L deionised H₂O; adjust pH to 5.8 with KOH; and filter-sterilise. Store in aliquots of 50 mL at -20°C in the dark.

7. 2,4-D stock solution: Prepare 1 mg/mL stock solution by completely dissolving 100 mg 2,4-D in a glass beaker with 250–500 μL 1 M KOH over a gentle heat source in a fume hood. Add 25 mL sterile deionised H_2O to the beaker, transfer to a new glass beaker, rinse the original beaker three times with 25 mL H_2O , and mix well the final solution (100 mL). Store in aliquots of 50 mL at -20°C in the dark.
8. Ethylenediaminetetraacetic acid ferric sodium salt (Fe-EDTA, Sigma) stock solution: Prepare 100 $\times$ stock with 4 g $\text{C}_{10}\text{H}_{12}\text{FeN}_2\text{NaO}_8$ in a final volume of 1 L deionised H_2O . Store at 4°C in the dark.
9. Timentin stock solution: Prepare 320 mg/mL stock solution by dissolving 3.2 g timentin (Duchefa) in 10 mL sterile deionised H_2O . Store in 1-mL aliquots at -20°C in the dark for up to 2 months.
10. Kinetin stock solution: Prepare 0.1 mg/mL stock solution with 5 mL kinetin (Sigma, 1 mg/mL) in 45 mL sterile deionised H_2O . Store in 15-mL aliquots at 4°C in the dark.
11. Basic solid medium for callus culture (MSB3): Dissolve 100 mL 10 $\times$ macro MS salt solution, 10 mL 100 $\times$ micro MS salt solution, 10 mL 100 $\times$ Fe-EDTA solution, 30 g sucrose, and 2.5 mL 2,4-D solution (1 mg/mL stock) in 990 mL deionised H_2O . Add 2 g Phytigel, adjust pH to 5.8, and autoclave. When the medium has cooled down, add 10 mL 100 $\times$ M5 vitamin solution and aliquot medium into Petri dishes under sterile conditions. Store at 4°C in the dark (see Notes 4 and 5).
12. Callus proliferation medium (MSB3 + Cu0.6): Before autoclaving, add 600 μL CuSO_4 solution (1 mg/mL stock) to basic MSB3 medium.
13. Co-culture medium (MSB3 + AS60): After autoclaving, add 2 mL AS solution (30 mg/mL stock; see Subheading 2.2) to basic MSB3 medium.
14. Callus selection medium (MSB3 + Cu0.6 + H40 + T225): Before autoclaving, add 600 μL CuSO_4 solution (1 mg/mL stock) to basic MSB3 medium and after autoclaving, supplement with 800 μL hygromycin B (50 mg/mL stock, Roche) and 700 μL timentin (320 mg/mL stock). To produce medium MSB3 + Cu0.6 + H30 + T225—used for dual selection—supplement medium with 600 μL hygromycin B (50 mg/mL stock) instead of 800 μL (see Note 6).
15. Plant regeneration medium (MSR26 + H20 + T225): Dissolve 100 mL 10 $\times$ macro MS salt solution, 10 mL 100 $\times$ micro MS salt solution, 10 mL 100 $\times$ Fe-EDTA solution, and 30 g sucrose in 990 mL deionised H_2O . Add 2 g Phytigel, adjust pH to 5.8, and autoclave. When the medium has cooled down, add 2 mL kinetin (0.1 mg/mL stock), 10 mL 100 $\times$ M5 vitamin

solution, 400 μ L hygromycin B (50 mg/mL stock), and 700 μ L timentin (320 mg/mL stock) and transfer 25-mL aliquots into Petri dishes under sterile conditions. Store at 4°C in the dark.

16. Plantlet germination medium (MSR63+H15+Ch4+T112): Dissolve 40 mL 10 $\times$ macro MS salt solution, 40 mL 100 $\times$ micro MS salt solution, 10 mL 100 $\times$ Fe-EDTA solution, and 10 g sucrose in 990 mL deionised H₂O. Add 6 g agar, 2 g Phytigel, and 4 g charcoal; adjust pH to 5.8; and autoclave. When the medium has cooled down, add 10 mL 100 $\times$ B5 vitamin solution, 300 μ L hygromycin B (50 mg/mL stock), and 350 μ L of timentin (320 mg/mL stock). Under sterile conditions, pipette 10 mL medium into glass tubes kept at a 45° angle until media is solidified. To produce medium MSR63+Ch4+T112—used for dual selection—omit to add hygromycin B to the plantlet regeneration medium described above. Store at 4°C in the dark (see Note 6).

3. Methods

3.1. Production of Immature Embryos

Steps 3 and 4 are conducted under sterile conditions.

1. Soak mature seeds of wild-type Bd21 plants in a Petri dish containing sterile deionised water for 2 h at room temperature.
2. Detach the top glume (lemma) from the seeds and collect seeds in water. Before sterilisation, remove all excess water.
3. Sterilise approximately 20 seeds in a Petri dish containing 20 mL 70% ethanol for 30 s. Remove ethanol and rinse once with sterile deionised water. Add 20 mL 1.3% sodium hypochlorite solution and gently shake the seeds for 4 min. Finally, rinse the seeds three times with sterile deionised water.
4. Place 20 seeds on top of two layers of sterile filter paper soaked with sterile deionised water inside a Petri dish. To synchronise germination, vernalise seeds for 2 days in the dark at 5°C, followed by 5–7 days at 25°C (2 days in the dark then 3–5 days with a 16-h photoperiod).
5. Pot germinated seedlings in a 2 $\times$ 2-cm cell tray containing the pre-watered compost. Grow plants in a Controlled Environment Room (CER) at 22°C with a 20-h photoperiod and cover seedlings initially with a propagator lid for 1–2 weeks.
6. When plants are starting to tiller, transfer plants together with their compost into “Roottrainer” (6) pots (4 $\times$ 5 $\times$ 20 cm) containing the pre-watered compost. Maintain plants in a CER at 22°C with a 20-h photoperiod or transfer to a glasshouse with the same environmental conditions. There is no need to add fertiliser to the compost used for plant culture.

7. To produce immature embryos, collect tillers from 7- to 9-week-old plants (see Subheading 3.2)
8. To establish seed stocks, stop watering plants after approximately 2–3 months when seeds are fully mature. Dry plants for approximately 2–4 weeks. Collect spikelets from each plant and store at 1.5°C, 7–10% humidity in the dark. Seeds can be kept for more than 10 years under these conditions.

3.2. Production of Compact Embryogenic Callus from Immature Embryos

1. Harvest tillers from 7- to 9-week-old *Brachypodium* plants when the immature seeds are swollen but still green.
2. Select immature seeds with a soft endosperm and remove the top glume (lemma) from the seeds while collecting the seeds in water. Before sterilisation, drain the seeds well.
3. Sterilise approximately 20 seeds for 30 s with 20 mL 70% ethanol in a sterile Petri dish covered with a lid. Drain ethanol and rinse the seeds with sterile deionised water. Add 20 mL 1.3% sodium hypochlorite solution and gently shake the seeds for 4 min. Rinse the seeds three times with sterile deionised water.
4. Isolate immature embryos, up to and including 0.3 mm in length, from the seeds. Homogeneous compact embryogenic callus (CEC) is produced at high frequency only from very small immature translucent embryos.
5. Culture immature embryos, with the scutellum facing up (i.e. embryo axis in contact with medium), onto MSB3+Cu0.6 solid medium and maintain for 3 weeks at 25°C in the dark. CuSO_4 significantly improves the growth and embryogenicity of CEC.
6. During the first 2–3 days of culture, remove under sterile conditions the shoots elongating from the embryos in order to help callus formation.
7. At week 3, split CECs with a creamy colour and pearly surface into one to three pieces. Discard all non-CEC tissue and transfer pieces of CEC onto fresh MSB3+Cu0.6 solid medium for a further 2 weeks at 25°C in the dark.
8. At week 5, divide CECs with a creamy colour and pearly surface into four to six pieces. Discard all non-CEC tissue and transfer pieces of CEC onto fresh MSB3+Cu0.6 solid medium for an additional week at 25°C in the dark.
9. At week 6, i.e. on the day of transformation, split CECs for the last time into four to six pieces and place CEC pieces onto fresh MSB3+Cu0.6 solid medium (50 calli/plate) for inoculation with *Agrobacterium*. In general, two plates (100 CECs) are sufficient for a small-scale transformation experiment aiming at the analysis of a gene construct.

3.3. *Agrobacterium*-Mediated Transformation of Compact Embryogenic Callus

1. Inoculate 5 μ L glycerol stock of *Agrobacterium tumefaciens* strain AGL1 (harbouring a binary transformation vector, e.g. pVec8-GFP) into 1 mL liquid LB + S50 medium (see Note 3).
2. Grow the *Agrobacterium* suspension overnight in an incubator shaker at 28°C and 200 rpm. Check that *Agrobacterium* is not grown at temperatures exceeding 28°C as this could lead to plasmid elimination.
3. Plate 200 μ L bacterial overnight culture onto solid MGL + S50 + AS30 medium using a sterile spreader. Culture plates upside down for 2 days at 28°C in the dark.
4. Scrape *Agrobacterium* layer from approximately half of a plate with an L-shaped sterile glass Pasteur pipette and add to a 50-mL disposable sterile tube containing 10 mL MSC + AS45 liquid medium. Strongly shake the tube by hand to fully suspend *Agrobacterium* aggregates.
5. Disperse *Agrobacterium* by shaking the suspension at 220 rpm for 45–60 min in a 28°C incubator.
6. Measure optical density of the bacterial suspension at $\lambda = 600$ nm and dilute to O.D.₆₀₀ = 1 using MSC + AS45 liquid medium.
7. Flood plates containing approximately 50 CECs by gently pipetting 13 mL *Agrobacterium* suspension (O.D.₆₀₀ = 1) onto the plates and suspend calli using an L-shaped sterile glass Pasteur pipette. Inoculate for 5 min at room temperature in a laminar flow hood.
8. Remove the bacterial suspension from the CEC plates with a pipette. Hand-pick each callus piece without a drying step and place straight onto a 9-cm sterile filter paper wetted with 500 μ L MSC + AS45 liquid medium. To prevent excessive drying of the calli, seal the Petri dish immediately with cling film and co-culture CECs for 2 days at 25°C in the dark. When setting up the transformation for the first time, an alternative co-culture procedure on solid co-culture medium can be used (see Note 7).

3.4. Selection of Transformed Calli

1. Transfer the co-cultured CECs onto MSB3 + Cu0.6 + H40 + T225 solid medium and maintain for 2.5 weeks at 25°C in the dark.
2. Dissect compact embryogenic sectors from each originally inoculated CEC and discard brown and/or soft non-embryogenic callus. Transfer pieces of CEC onto fresh MSB3 + Cu0.6 + H40 + T225 solid medium for an additional 2.5 weeks at 25°C in the dark. At this stage, all CEC pieces dissected from one transformed callus are kept together. An alternative protocol combining hygromycin selection and green fluorescent (GFP) screening can also be implemented for large-scale transformation (see Table 2 and Note 6).

Table 2
Summary of selection schemes

	Chemical selection (hygromycin) only	Chemical selection (hygromycin) and visual screening (GFP) (see Note 6)
Callus selection I (Subheading 3.4 step 1)	• MSB3 + Cu0.6 + H40 + T225 • 2.5 weeks • Keep CEC—discard brown and/or soft non-embryogenic callus	• MSB3 + Cu0.6 + H40 + T225 • 3 weeks • Select GFP + CEC sectors only
Callus selection II (Subheading 3.4 step 2)	• MSB3 + Cu0.6 + H40 + T225 • 2.5 weeks • Keep CEC—discard brown and/or soft non-embryogenic callus	• MSB3 + Cu0.6 + H30 + T225 • 3 weeks • Select GFP + CEC sectors only
Plant regeneration (Subheading 3.5 step 1)	• MSR26 + H20 + T225 • 2–3 weeks • Use shoots (rooted or not) for germination	• MSR26 + H20 + T225 • 2–3 weeks • Use GFP + shoots (rooted or not) for germination
Plant germination (Subheading 3.5 step 2)	• MSR63 + H15 + Ch4 + T112 • 2–3 weeks • Transfer rooted plantlets into soil	• MSR63 + Ch4 + T112 • 2–3 weeks • Transfer GFP + rooted plantlets into soil

**3.5. Regeneration
of Transgenic Plants**

1. Five weeks after transformation, transfer hygromycin-resistant calli onto regeneration medium MSR26 + H20 + T225 for 2–3 weeks at 25°C under 16-h photoperiod. Keep all callus pieces from the same independent T-DNA line together for regeneration.
2. Seven to eight weeks after transformation, transfer shoots (rooted or not) to tubes containing germination medium MSR63 + H15 + Ch4 + T112. Culture for 2–3 weeks at 25°C under 16-h photoperiod. An alternative germination medium—without hygromycin—is used when applying dual hygromycin and GFP selection (see Note 6).

**3.6. Seed Production
from T-DNA Plants**

1. Pot fully rooted hygromycin-resistant plantlets into 2 × 2-cm cell trays containing pre-watered compost. Grow plants in a CER at 22°C with a 20-h photoperiod. To ensure adaptation of plantlets to low humidity conditions, cover seedlings with a propagator lid for 1–2 weeks.
2. When plants start to tiller, transfer plants together with their original compost into “Roottrainer” pots containing pre-watered compost. Grow plants in a CER or glasshouse at 22°C with a 20-h photoperiod for 5–6 weeks.

3. When the seeds are fully mature (2–3 months after potting into soil), cease watering plants and dry plants for approximately 2–4 weeks. Collect spikelets from each plant and store at 1.5°C, 7–10% humidity in the dark. Seeds can be stored for more than 10 years under these conditions.

4. Notes

1. All solutions and culture media to be kept in the dark are stored wrapped with aluminium foil to prevent exposure to light. Unless stated otherwise, all stock solutions are stored at 4°C for up to 3 months and at –20°C for up to 1 year. Stocks stored at –20°C should not be frequently freeze-thawed.
2. The antibiotic used for bacteria selection depends upon the type of binary vector used for transformation. As an example, add 50 µL kanamycin (100 mg/mL stock)—instead of streptomycin—to all bacterial culture media when pCLEAN (11) or pBrachyTAG binary vectors are used instead of pVec8-GFP.
3. The presence of the Ti plasmid and the binary vector in the *Agrobacterium* strain to be used for plant transformation should be verified by performing PCRs with plasmid-specific oligonucleotide primer pairs. In addition, *Agrobacterium* strains should be assessed via a Benedict's test to confirm that no *Escherichia coli* contamination has occurred (i.e. streak an *Agrobacterium* strain onto LYM solid medium, grow for 2–3 days at 28°C, and cover with 0.5–1 mL Benedict's test solution. The blue solution will turn yellow in the presence of *Agrobacterium* growing on LYM).
4. For all 1 L stock solutions, dissolve all components in 0.8 L deionised H₂O, mix using a magnetic bar and stirring plate, then adjust to 1-L final volume using a graduated cylinder. For all plant tissue culture media, however, adjust to a final volume of 990 mL, add the gelling agent, and after autoclaving, supplement with 10 mL vitamin stock solution.
5. All culture media are autoclaved using a 90-min total cycle, including 15 min at 120°C. Solid media is poured in approximately 25-mL aliquots into sterile single-use Petri dishes, and piles of closed Petri dishes are left to dry under the laminar flow hood for 6–8 h or overnight to avoid accumulation of condensation on the lid or surface of the medium. Stacks of 20 plates are then sealed using cling film. Plant tissue culture medium can be stored for up to 1 month at 4°C.
6. To perform dual selection, screen CECs growing on hygromycin (see Subheading 3.4 step 1) 3 weeks after transformation

for the presence of small bright green fluorescent (GFP) sectors using a stereomicroscope with a UV light source. Dissect each fluorescent sector and culture them as independent T-DNA lines onto MSB3+Cu0.6+H30+T225 solid medium for additional 3 weeks at 25°C in the dark (6). Six weeks after transformation, screen calli for green fluorescence and transfer hygromycin-resistant and GFP+ calli onto regeneration medium MSR26+H20+T225 for 2–3 weeks at 25°C under 16-h photoperiod. At this stage, keep all GFP+ callus pieces from the same independent callus line together (6). Eight to nine weeks after transformation, transfer shoots (rooted or not) to tubes containing germination medium MSR63+Ch4+T112. Culture for 2–3 weeks at 25°C under 16-h photoperiod. Ten to eleven weeks after transformation, confirm that plantlets in tubes are GFP+ before transferring into soil mixture (see Subheading 3.6 step 1). GFP fluorescence is easily detected in the roots of plantlets.

7. The co-culture step can be undertaken on solid medium instead of filter paper. The former procedure is generally slightly less efficient but prevents variability of results linked to excessive drying of CECs on filter paper. This approach is recommended when setting up the transformation process for the first time as it is more foolproof. To perform the co-culture on solid medium, remove the bacterial suspension from the inoculated CECs (Subheading 3.3 step 7), hand-pick the calli, and place them onto a dry sterile filter paper. Perform a desiccation treatment by leaving the CECs to dry uncovered for 7 min under the laminar flow hood. Subsequently, place the CECs onto MSB3+AS60 medium for co-cultivation (2 days at 25°C in the dark) and then proceed to Subheading 3.4 step 1.

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Chapter 13

Transformation of Barley (*Hordeum vulgare* L.) by *Agrobacterium tumefaciens* Infection of In Vitro Cultured Ovules

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Abstract

Agrobacterium-mediated transformation of in vitro cultured barley ovules is an attractive alternative to well-established barley transformation methods of immature embryos. The ovule culture system can be used for transformation with and without selection and has successfully been used to transform cultivars other than Golden Promise indicating minor genotype dependency. The method allows for the rapid and direct generation of high-quality transgenic plants where the transgenes are stably expressed and show Mendelian inheritance in subsequent generations.

Key words: *Agrobacterium tumefaciens*, Barley cultivars, Ovule culture, Transformation, Zygote

1. Introduction

Efficient protocols for *Agrobacterium*-mediated transformation of barley immature embryos have been developed, are widely used, and are currently improved (1–3). However, it is a major drawback that immature embryos of many barley cultivars show poor regeneration ability. In consequence, the cultivar Golden Promise with its very high regeneration ability is used in almost all published barley transformation studies.

The zygote within the in vitro cultured barley ovule is an attractive alternative target for *Agrobacterium*-mediated transformation because the regeneration ability of this target is less genotype dependent than the immature embryo and because there is a direct development from zygotes into plantlets without an intervening dedifferentiated callus phase (4, 5). The barley ovule culture technique that allows for the direct regeneration of plants from

the zygotes was established by Holm and coworkers in 1995 (6). Ovules can be isolated from 1 to 30 h after pollination and cultured in vitro. Within the next 3 weeks of culture, about half of the ovules give rise to embryos. The embryos germinate into plantlets at frequencies of 75–90% after an additional 2–3 weeks on regeneration medium. Many ovules contain more than one embryo with an average of 1.4–1.5 individual embryos formed per responding ovule. The individual embryos are either fully separated or present as aggregates that in most cases can be easily separated. However, in some instances, the aggregated embryos share the same shoot or root meristem.

The protocol of generating transgenic plants from in vitro cultured barley ovules infected with *Agrobacterium* is described below. In barley, the egg cell is fertilized about 45 min after pollination, the S phase is initiated 12 h after pollination, and the first division of the barley zygote takes place 22–29 h after pollination (7–9). If the transgene is inserted into the host genome prior to or early in S phase, all derived cells will be transgenic while post-S-phase integrations will lead to segregation for the trait between the two daughter cells of the zygote. In the present protocol, the time used for ovule isolation and infection after hand pollination and fertilization is limited to 2–3 h, leaving 9–10 h for T-DNA transfer and integration before initiation of the S phase. When using the gene for the green fluorescence protein (GFP) for transformation, we nearly always find that the stably transformed, nonchimeric GFP-expressing embryos develop together with nontransformed embryos. Hence, given the high frequency of polyembryo formation within in vitro cultured ovules, the time of integration may range from the zygotic stages until an early stage of embryo formation where a transformed cell from early stage embryos develop into a new partly or completely individualized embryo.

The transformation efficiencies obtained show that the ovule culture transformation technique is a promising alternative to other transformation techniques currently used in barley. When implementing hygromycin selection, we obtain transformation frequencies of 3.4 transgenic plants per 100 infected ovules using the cultivar Golden Promise. Regeneration of transgenic plants without selection occurs at an average frequency of 0.8% (4). We obtained transformation frequencies of 2.2, 1.4, 1.4, and 1, respectively, in four barley cultivars Femina, Salome, Corniche, and Alexis known to have poor response in other types of tissue culture. Although these transformation frequencies were lower than for Golden Promise, the differences were not statistically significant (5). Besides being less genotype dependent, the ovule culture system is a fast transformation system requiring only 6–9 weeks of in vitro culture compared to 10–18 weeks for immature embryo transformation. The ovule-derived plants are of high quality and are fully fertile showing little somaclonal variation, and the transgenes are stably expressed and inherited.

2. Materials

2.1. Growth Conditions of Donor Plants

1. Seed of spring barley is sown at weekly intervals in 2-L pots with a growth mix containing fertilized Unimuld (Pindstrup Moseby A/S, Denmark) and Grodan® (Rockwool® Group, Denmark) at 8:1.
2. Plants are grown in growth cabinets with temperatures of 15°C at day and 10°C at night with a 16-h light period. The light is provided by halogen-metal lamps at 250 W daylight with an intensity of 350 $\mu\text{mol}/\text{m}^2/\text{s}$ at the level of the pot surface. The relative humidity in the growth cabinet is 75–80% (see Note 1).

2.2. *Agrobacterium* Strain and Vectors

Agrobacterium tumefaciens strain AGL0 (10) is used together with a vector. Presently, we have used the protocol with the binary vectors pWBVec8 containing the GFP gene or the GUS gene (11) or with the twin T-DNA vector pWBVec82b (12) with an antisense C-hordein gene. Both vectors contain the hygromycin resistance gene as selection gene.

2.3. Preparation of *Agrobacterium* Glycerol Stocks

1. Streak *A. tumefaciens* AGL0 carrying the vector on solid MG/L medium supplemented with 25 mg/L rifampicin and appropriate antibiotics. Grow at 28°C in the dark for 2–3 days until single colonies are clearly visible.
2. Inoculate 5 mL MG/L liquid medium supplemented with 25 mg/L rifampicin and appropriate antibiotics with a single colony. Grow the culture for 24–40 h at 28°C in the dark with shaking at 180 rpm until an OD_{600} of around 1.0.
3. Mix 200 μL of the bacterial culture and 200 μL of 30% aqueous glycerol in an Eppendorf tube. Vortex and maintain at room temperature for 2–4 h turning the tubes upside down every half an hour.
4. Store the stocks at -80°C .

2.4. Media Used in the Protocol

Except otherwise described, all media components are supplied by Sigma-Aldrich, and all media and stocks are made up using Milli-Q water.

2.4.1. Bacterial Culture Medium

The bacterial culture medium is MG/L (1): 5.0 g/L tryptone, 5.0 g/L mannitol, 2.5 g/L yeast extract, 1.0 g/L l-glutamic acid, 250 mg/L KH_2PO_4 , 100 mg/L NaCl, and 100 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The medium is adjusted to pH 7.0 with NaOH. For solid medium, 15 g/L agar is added. After autoclaving, 1 $\mu\text{g}/\text{L}$ biotin is added.

2.4.2. Plant Tissue Culture Media

Four different plant culture media are used in this protocol: cocultivation medium, induction medium, regeneration medium, and

rooting medium. Additionally, an inoculation medium for *Agrobacterium* is used.

1. Cocultivation medium: 2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3) (Duchefa, M0238), 165 mg/L NH_4NO_3 , 1 g/L casein hydrolysate, 690 mg/L proline, 150 mg/L myo-inositol, 90 g/L maltose, 1 mg/L BAP, 100 μM (20 mg/L) acetosyringone (Aldrich, D13-440-6), 10 mL/L Kao vitamins (Kao and Michayluk vitamin solution (100 $\times$), Sigma, B3129), and 6 g/L Phytigel. The pH is adjusted to 5.8 with NaOH.
2. Induction medium: Contains the same components as the cocultivation medium but lacks acetosyringone, and the medium is supplemented with the antibiotic Timentin (Ticarcillin/Clavulanic acid (15:1), Duchefa, T0190) at 150 mg/L and 50 mg/L hygromycin B (Duchefa, H 0192).
3. Regeneration medium: 2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3) (Duchefa, M0238), 165 mg/L NH_4NO_3 , 250 mg/L casein hydrolysate, 750 mg/L glutamine, 0.4 mg/L BAP, 30 g/L maltose, 10 mL/L Kao vitamins (Kao and Michayluk vitamin solution (100 $\times$), Sigma, B3129), 3.5 g/L Phytigel, and 50 mg/L hygromycin. The pH is adjusted to 5.8 with NaOH.
4. Rooting medium: 2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3) (Duchefa, M0238), 165 mg/L NH_4NO_3 , 250 mg/L casein hydrolysate, 146 mg/L glutamine, 30 g/L maltose, 3.5 g/L Phytigel, and 50 mg/L hygromycin. The pH is adjusted to 5.8 with NaOH.

The inoculation medium contained the same compounds as the cocultivation medium but without the addition of Phytigel.

2.4.3. Supplements to the Media

Kao vitamins (100 $\times$ stock): Purchased as sterile 100 $\times$ stock and stored at -20°C .

Acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) (100 mM stocks): Dissolve in 70% ethanol to give 100 mM stock solution (20 mg/mL). Store at -20°C in 1-mL aliquot.

BAP (1 mg/mL stocks): Dissolved in a small volume of 1 M NaOH and make up to volume with water, mix well, and store at -20°C in 1-mL aliquot.

Hygromycin B (50 mg/mL stock): Purchased as a sterile 406 mg/mL stock, divide into 50-mg aliquots, and stored at 4°C .

Timentin (Ticarcillin/Clavulanic (15:1)) (150 mg/L stock): Dissolve in water to give 150 mg/mL, filter sterilize, and store at -20°C in 1-mL aliquot.

Biotin (1 mg/100 mL stock): Dissolve in water, filter sterilize, and store at -20°C in 1-mL aliquots (add 100 μL to 1 L MG/L).

Silwet L-77 (Lehle seeds, USA, VIS-01) (1% v/v stock): Dissolve in water, filter sterilize, and store at 4°C in 0.5-mL aliquots.

2.5. Tissue Culture Media Preparation

Maltose is dissolved in water at double concentration and autoclaved separately. The other media components except for the Kao vitamins are mixed together at double concentration, pH adjusted, and autoclaved. After autoclaving and cooling, the two solutions are mixed, and the sterile Kao vitamins are added. At this stage, the *vir*-gene inducer acetosyringone is added to the cocultivation medium, the antibiotic Timentin is added to the induction medium, and the selection agent hygromycin is added to the induction medium, the regeneration medium, and the rooting medium. The cocultivation, induction, and regeneration medium are poured into 5-cm-deep Sterilin Petri dishes (Barloworld Scientific Ltd. UK, 798105-124), and the rooting medium is poured into jars (4.5 cm × 11 cm, Greiner vials, in vitro A/S 960 161).

2.6. Other Equipment

1. Scissors, forceps (Fine Science Tools, FST # 11200-33), and nail clipper for emasculation.
2. Two fine forceps for ovule isolation (Fine Science Tools, FST # 11254-20).
3. Binocular microscope with light in the microscope platform (Zeiss, Stemi 2000-C).
4. Syringe with needle for *Agrobacterium* infection (syringe 1 mL, Becton Dickinson, 300013; needle 0.4 mm × 19 mm, Becton Dickinson, 3002200).

3. Methods

3.1. Emasculation of Barley Spikes

1. Emasculate spikes 3–4 days before anthesis (see Note 2) (Fig. 1a).
2. Cover the emasculated spike with a paperbag until pollination 4–5 days later.

3.2. Preparation of *A. tumefaciens* Overnight Cultures

1. The day before pollination, ovule isolation, and infection, take 200 µL of an *Agrobacterium* glycerol stock and add to 5 mL MG/L liquid medium without antibiotics in a 50-mL centrifuge tube. Incubate the culture overnight at 28°C with shaking at 180 rpm (around 18 h).
2. Add acetosyringone to a final concentration of 100 µM to the 18-h overnight culture. Put the culture back on the shaker for 3–4 h.

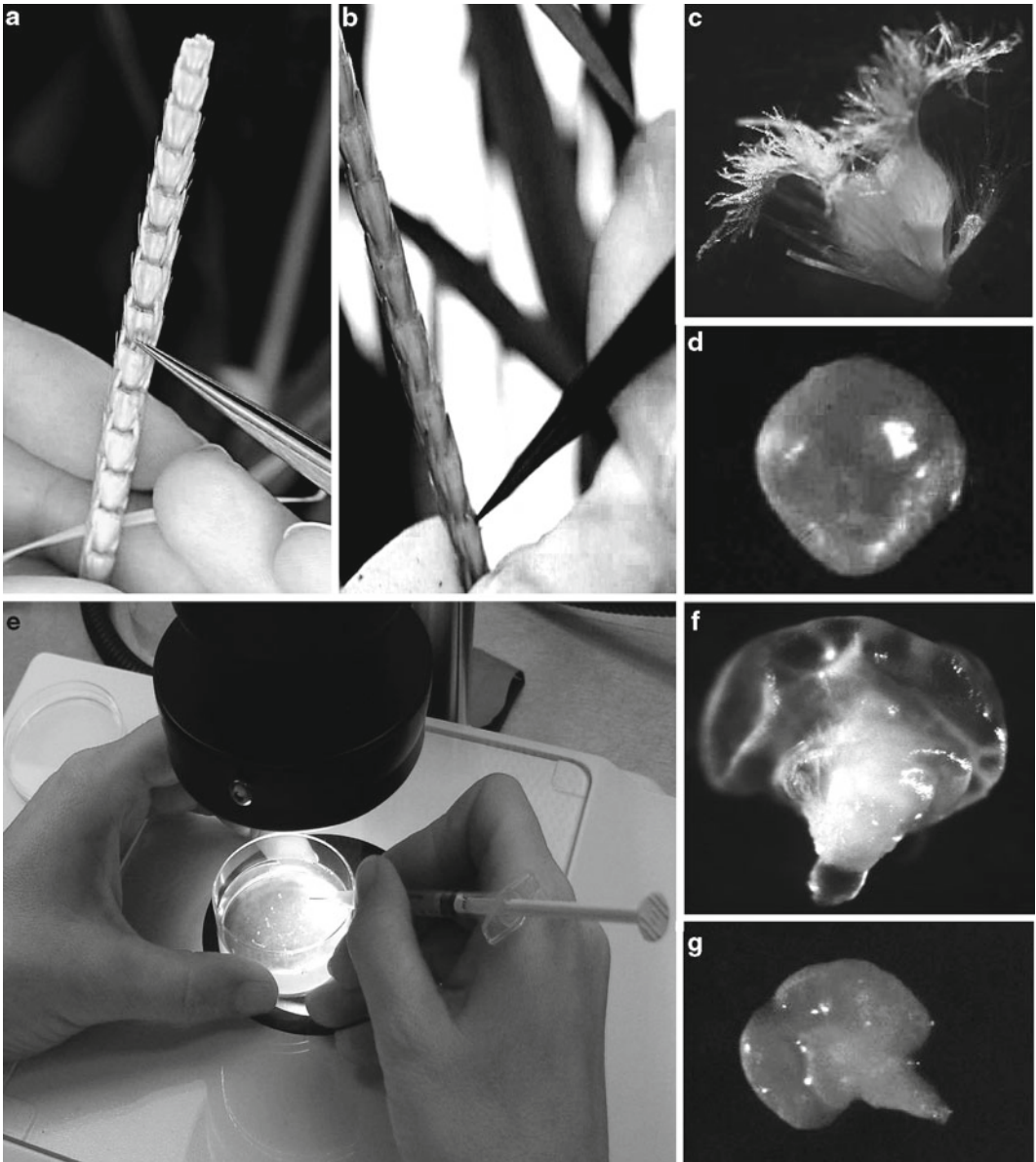


Fig. 1. (a) Emasculation: Removal of immature anthers after cutting off the awns and the upper part of the bracts just above the anthers. (b) Pollination: Insertion of one to two anthers with mature pollen into the florets of emasculated spikes. (c) Isolated ovary with the two lodicules still attached. (d) Freshly isolated ovule. (e) Infection of freshly isolated ovules with a fine needle on a syringe filled with *Agrobacterium* inoculum. (f) Embryo developed within the ovule 14 days after isolation and infection. (g) Embryo excised from an ovule 16 days after isolation and infection.

3.3. Hand Pollination and Isolation of Barley Ovaries

3.3.1. Pollination

1. Collect anthers with mature pollen on the day of pollination, ovule isolation, and infection.
2. Place one to two of the freshly harvested anthers into each floret of the emasculated spikes with the forceps (Fig. 1b). We usually pollinate three to four spikes at a time to make sure that we can isolate around 50 ovules.

3.3.2. Collection of Fertilized Spikes and Sterilization of Ovaries

1. Cut off the hand-pollinated spikes 1–1.5 h after pollination and place them in tap water until ovary isolation.
2. Remove the ovaries from the florets with the two lodicules still attached (see Note 3) (Fig. 1c). Place the ovaries in a 9% sterile maltose solution in Sterilin 5-cm Petri dishes. From hereon, all manipulations are performed in a laminar flow hood under sterile conditions.
3. Surface sterilize the ovaries for 10 min in 15 mL 1.5% hypochlorite (VWR, no. 27900.296) with 10 μ L of Tween 20 (Sigma, P-1379) per 50 mL hypochlorite solution. Rinse the ovaries three times in sterile water and transfer them to liquid 9% maltose (see Note 4).

3.4. Isolation of Ovules

1. Excise the ovules under a dissection microscope using fine-tipped forceps (see Note 5). Place the ovary with the placental side facing downwards on an empty Petri dish on the microscope platform. Fix the ovary by placing a pair of fine forceps into the upper part (stigma part) of the ovary and remove the pedicel and the two lodicules with the second pair of forceps.
2. While still fixed, open the lower part of ovaries where the ovule is situated with the second pair of forceps. Cut the ovarian tissue with the tip of the forceps just above the ovule and along one side down to the micropylar end taking care not to cut the ovule. Fold back the triangle to expose the ovule and gently slide the tip of the forceps underneath the ovule to release it from the placenta connection and lift the ovule out (see Note 6).
3. Place eight to ten ovules on the cocultivation medium with their placental side facing downwards (Fig. 1d).

3.5. *Agrobacterium* Infection

1. Infect the ovules with *Agrobacterium* after isolation of around 50 ovules (2–3 h) (see Note 7). Take the centrifuge tube with the overnight *Agrobacterium* culture supplemented with acetosyringone from the shaker. Pellet the *Agrobacterium* cells by centrifugation at $2,000 \times g$ for 7 min and resuspend the pellet in inoculation medium to an OD₆₀₀ of around 1.7. Add Silwet L-77 to the *Agrobacterium* suspension to a final concentration of 0.05%.
2. Fill a 1-mL sterile syringe with the *Agrobacterium* inoculum and screw a sterile fine needle (0.4 mm $\times$ 9 mm) on the syringe. Punch a hole with the fine needle in the ovule wall near the micropyle and place a small droplet of *Agrobacterium* inoculum on top of the perforated area (see Note 8) (Fig. 1e).
3. Transfer the infected ovules with their placental side facing downwards to Petri dishes with fresh cocultivation medium.
4. Incubate the cultures at 23°C in the dark for 18–20 h.

3.6. Transformation with Selection

3.6.1. Embryo Development

1. After 18–20 h of cocultivation, transfer the ovules to induction medium containing Timentin to kill *Agrobacterium* and hygromycin as selective agent. Place the ovules with their placental side facing downwards (see Note 9).
2. Incubate the cultures at 23°C in the dark. Embryos of 1–2 mm will develop within the ovules after 2–3 weeks (see Note 10) (Fig. 1f).

3.6.2. Regeneration of Transgenic Plants

1. Isolate and transfer the embryos to Petri dishes with regeneration medium containing hygromycin as selection agent (Fig. 1g) (see Note 11).
2. Transfer plantlets as they reach a size of 2–3 cm to jars with 25 mL of rooting medium containing hygromycin as selection agent during the next 3 weeks.
3. Transformed plantlets will form roots 1–3 weeks after transfer to rooting medium. Plantlets with at least three strong roots are transferred to soil.
4. Initially, pot up the plantlets individually into 7 × 7 × 8-cm pots and place them for 1–2 weeks under plastic to maintain humidity and to acclimate the plantlets to greenhouse conditions.
5. Transfer the plants into 2-L pots after 3–4 weeks. Here, they grow to maturity.

3.7. Transformation Without Selection

For transformation without selection, the procedure is the same except that the ovules are grown on induction, regeneration, and rooting medium without the selection agent hygromycin.

3.8. Identification of Transgenic Plants

When the plants have three to five shoots, young leaf material is collected for PCR and Southern blot analysis to confirm the presence of the transgenes. Transformation with selection gives very few chimeric transformed plants, and plant material can be collected from just one shoot. But without selection, it is our experience that at least one out of three transformants is chimeric, and we therefore analyze the plants obtained from transformation without selection for chimeras in the following way:

1. Collect young leaf material for DNA isolation from at least three shoots evenly spread in each mature plant.
2. Isolate the DNA from each of the three samples. Adjust to the same DNA concentration and make a mix of the three samples.
3. PCR-analyze the DNA mix to assess which plants are transgenic.
4. For the transgenic plants identified, PCR-analyze the three DNA isolations individually to assess which shoots are transgenic.

5. If the transgenic plant is chimeric, collect leaf material from shoots in the vicinity of the transgenic shoots for DNA isolation and PCR analysis to identify the transformed shoots of the plants.
6. Harvest seeds only on transgenic shoots.

3.9. Estimation of Transformation Efficiency

1. The transformation frequency is estimated as the number of Southern blot hybridization confirmed transformed plants per 100 ovules infected.
2. The number of confirmed transformed plants per 100 regenerated plants is also estimated when transforming without selection. This number shows how many plants that have to be screened in order to identify one transgenic plant (see Note 12).

4. Notes

1. Avoid spraying of the donor plants 2 weeks before the use of spikes for emasculation or pollination.
2. We emasculate in this way: Cut off the awns of the spike with scissors, remove the upper and lower two to three florets on each site of the spike, and cut the bracts of the remaining florets on the spike just above the anthers with a nail clipper. The three anthers can then easily be removed with the forceps without hurting the stigma of the ovary.
3. We isolate the ovaries in this way: Leave the florets on the spike. Expose the ovaries of each floret by slicing the lemma at each site with the tip of the fine forceps and remove the adaxial site of the lemma. Insert the forceps behind the ovary, glide it down towards the lodicules, and carefully drag out the ovary with the two lodicules still attached. Isolation of ovaries with the lodicules still attached ensures that the tip of the ovules is still intact.
4. Storage of the ovaries in water makes the ovaries too watery.
5. A microscope with light in the platform is ideal for ovule isolation.
6. Ovules are easier to isolate if they are briefly transferred to a sterile filter paper placed on solid cocultivation medium to dry out some of the excess liquid. Transfer only a few ovules at a time to the sterile Petri dish on the microscope platform to avoid too much drying out by the microscope light.
7. It is important to use 0.6% Phytigel in the cocultivation medium since it quickly soaks away excess inoculum.
8. To remove excess inoculum on top of the ovules, the ovules can be turned upside down and gently moved along the medium before transfer to fresh Petri dishes with cocultivation medium.

9. Media with antibiotics should not be more than 1 week old.
10. Best selection is obtained when embryos of only 1–1.5 mm are transferred to regeneration medium, so that the fresh selection agent in the regeneration medium can reduce the growth of the nontransformed embryos more efficiently.
11. Identify embryos that have grown together within the ovule under the microscope and, if possible, gently divide them. This greatly reduces the number of transformed chimeras obtained.
12. In the experiments we have performed without selection, we obtain 0.6–1.1 transformed plants per 100 ovules and 2–2.4 transformed plants per 100 plants regenerated. This means that with the present protocol, around 50 plants regenerated without selection have to be tested for the presence of the transgene in order to identify one transgenic plant.

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Chapter 14

Biolistic-Mediated Production of Transgenic Oil Palm

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Abstract

The effectiveness of mannose (using phosphomannose isomerase [*pmi*] gene) as a positive selection agent to preferably allow the growth of transformed oil palm embryogenic calli was successfully evaluated. Using the above selection agent in combination with the previously optimized physical and biological parameters and the best constitutive promoter, oil palm embryogenic calli were transformed with *pmi* gene for producing transgenic plants. Bombarded embryogenic calli were exposed to embryogenic calli medium containing 30:0 g/L mannose to sucrose 3 weeks postbombardment. Selectively, proliferating embryogenic calli started to emerge around 6 months on the above selection medium. The proliferated embryogenic calli were individually isolated once they reached a specific size and regenerated to produce complete plantlets. The complete regenerated plantlets were evaluated for the presence of transgenes by PCR and Southern analyses.

Key words: Biolistics, Positive selection, Transgenic oil palm, *Elaeis guineensis*, Oil crop

1. Introduction

Malaysia is the largest exporter of palm oil in the world, and her crude palm oil production is increasing annually. In 2008, Malaysia produced 17.73 million tonnes of palm oil, which was 12.1% or 1.91 million tonnes higher than the production in 2007 (1). However, there are major problems faced by the oil palm industry in Malaysia: shortage of labor, scarcity of arable land, and fluctuations in the price of the commodity. Quite recently, the price of palm oil declined sharply (around 66% within a period of 8 months) from the highest recorded at RM 4,179 per tonne (approximately US\$ 1,155) in early March 2008 to the lowest at RM 1,403 (approximately US\$ 388) in late November 2008 (1). With the current premier position, it is essential for the oil palm industry to remain competitive by increasing yield per unit area as well as producing novel high-value products using approaches such as genetic engineering as opposed to the more time consuming

conventional breeding (2). Among the targeted high-value products for oil palm are oleic acid, stearic acid, palmitoleic acid, ricino-leic acid, biodegradable plastics, and carotenoids (3–5). Currently, oil palm genetic engineering is mainly carried out for research purposes and transgenic plants are being planted in fully contained biosafety screenhouses.

Plant genetic transformation involves the uptake of naked DNA by competent plant cells, followed by integration into the genome, and subsequent expression of the gene to synthesize the gene product (6). Prerequisites for producing transgenic plants are the availability of a suitable gene transformation system, the existence of an efficient transformant selection system and a regeneration system to fully regenerate transformed plant cells. A selection system enables transgenic cells or tissues to be isolated and selectively regenerated into a whole plant post transformation. The expression of a selectable marker gene results in a product that allows the survival of the transformed cells in the presence of a selective agent that prevents regeneration of the nontransformed cells (7). There are two types of selection systems: negative and positive selection systems (8). The most common negative selection agents are herbicides (such as phosphinothricin) or antibiotics (such as hygromycin and kanamycin). These selection agents are usually toxic to the untransformed cells and sometimes could affect transgenic plant regeneration.

In contrast, a positive selection system allows transformant identification by giving a selective advantage to genetically transformed cells, promoting their growth without directly damaging the nontransformed cells (8, 9). The transformed cells will have certain metabolic advantages for growth stimulation such as the capability to metabolize a rare sugar, respond to a particular hormone, or adapt to extreme temperature. Examples of positive selection agents are 2-deoxyglucose (*DOG^R1* gene), betaine aldehyde (*badh* gene), D-xylose (*xylA* gene), D-mannose (*manA/pmi* gene), benzyladenine-N-3-glucoronide (*uidA/gusA*), L-cysteine (*ocs* gene), methotrexate (*dhfr* gene), 4-methyltryptophan (*tdc* gene), and S-aminoethyl (*dhps* gene) (8).

This work was carried out to evaluate the D-mannose selection system in oil palm. Mannose will prevent cell growth and development when it is phosphorylated by hexokinase to mannose-6-phosphate. Accumulation of mannose-6-phosphate causes growth inhibition of the nontransgenic cells due to starvation with respect to intracellular phosphate and ATP (10). Cells transformed with the *pmi* gene will acquire the ability to convert mannose-6-phosphate to the metabolizable fructose-6-phosphate which enables these cells to survive on mannose-containing media (11).

Determination of the optimal concentration of mannose was carried out to identify the concentration required to select transformed from nontransformed cells. Using the optimal concentration

of selection agent in combination with the optimized physical and biological parameters affecting DNA delivery and an efficient promoter for expressing the transgene in oil palm cells, the *pmi* gene was bombarded into oil palm target tissues, embryogenic calli. Transformed embryogenic calli were selected using the optimal concentration of mannose and followed by proliferation and regeneration of transgenic plants. Molecular analyses were conducted to confirm the transgenic status of the plants.

2. Materials

2.1. Equipment

Biolistics—PDS-1000/He Apparatus (Bio-Rad), autoclave (Tomy), incubator shaker (Innova), electrophoresis unit and power supplies (Bio-Rad), PCR apparatus (MJ Research—PTC-100 and PTC-200), centrifuge RC5C *Plus* (Sorvall), vacuum chamber, hybridization oven (Techne Hybridiser HB-1D), vortex (Finemixer Sh2000, FinePCR), pH meter (Mettler Toledo), hot plate and stirrer, analytical balance, plant tissue-culture incubator (Convion TC-16), and microcentrifuge (Hettich Mikro120).

2.2. Supplies

Gold microcarriers (Bio-Rad), macrocarrier (Bio-Rad), stopping screen (Bio-Rad), rupture disk (Bio-Rad), QIAGEN Plasmid Maxi kit, Treff microtube (Switzerland), Petri dish, nylon membrane (Hybond-N+, Amersham), 3MM paper (Whatman), and X-ray film (Kodak). Chemicals from Sigma unless otherwise stated.

2.3. Media and Solutions

1. Bacterial LB medium: 10 g NaCl, 10 g tryptone (Pronadisa), and 5 g yeast extract (Pronadisa), pH 7.0 in 1 L distilled water.
2. GUS assay buffer: GUS assay buffer (0.1 M NaPO₄ buffer (pH 7.0) [mixture of NaH₂PO₄ and Na₂HPO₄], 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 0.01 M EDTA, 1 mg/mL X-gluc (5-Bromo-4-Chloro-3-Indolyl-β-D-glucuronide) (Phytotech), 1 μL/mL Triton-X, and 20% methanol (v/v) (Merck)). The GUS assay buffer was filter sterilized before used or stored at −20°C.
3. Callus initiation medium: MS salts (12), Y3 vitamins (13) (1 mg/mL thiamine-HCl, 1 mg/mL pyridoxine HCl, 1 mg/mL nicotinic acid), 0.1 mg/mL *myo*-Inositol, 0.1 mg/mL L-glutamine, 3% sucrose, 5 × 10^{−5} M 2,4-D, 0.25% activated charcoal, and 0.7% agar, pH 5.7 (22).
4. Embryogenic medium: MS macro- and micronutrients and Y3 vitamins supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-asparagine, 10 μM 2,4-D, 5 μM NAA, and 30 g/L sucrose (22).

5. Polyembryogenic culture medium: MS macro- and micronutrients and Y3 vitamins supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-arginine and L-asparagine, 5 μ M IBA, 0.7% agar, and 30 g/L mannose. Adjust to pH 5.7 with KOH prior to autoclaving (22).
6. Shoot induction medium: MS macro- and micronutrients, Y₃ vitamins supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-arginine and L-asparagine, 0.1 μ M NAA, 0.4% agar, and either 30 g/L mannose or sucrose. Adjust to pH 5.7 with KOH prior to autoclaving (22).
7. Liquid root induction medium: MS macro- and micronutrients and Y3 vitamins supplemented with 300 mg/L L-glutamine, 100 mg/L *myo*-inositol, 10 μ M 2,4-D, 70 μ M NAA, 0.15% activated charcoal, and either mannose or sucrose at 30 g/L. Adjust the medium to pH 5.7 with KOH prior to autoclaving (22).
8. CTAB buffer: 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 NaCl, 2% CTAB, 1% PVP, and 0.2% 2-mercaptoethanol (14).
9. PCR mixture: DNA (50 ng total DNA or 5 ng plasmid DNA), reaction buffer (50 mM KCL and 10 mM Tris-Cl pH 8.3) (Promega), dNTPs (200 μ M each) (Promega), 1.5 mM MgCl₂ (Promega), primers, and Taq DNA polymerase (Promega).
10. Washing buffer: 76% ethanol and 1 mM ammonium acetate.
11. TE buffer: 10 mM Tris-HCl pH 7.5 (10 mM Tris) and 1 mM EDTA.
12. TBE buffer: 45 mM Tris-Borate and 1 mM EDTA, pH 8.0.
13. Depurinating buffer: 0.2 M HCl.
14. Denaturing buffer: 1.5 M NaCl and 0.5 M NaOH.
15. Neutralization buffer: 1 M Tris-HCL (pH 8.0) and 1.5 M NaCl.
16. SSC buffer: 175.3 g NaCl and 88.2 g sodium citrate in 1 L, pH 7.0.
17. Prehybridization buffer: 40% pipes/NaCl pH 6.8 (1.5% pipes, 8.7% NaCl and 0.37% EDTA, pH 8.0), 20% Denhardt's 50 $\times$ (1% BSA, 1% Ficoll, 1% PVP, and 10% SDS) (Promega), 0.5% ssDNA 10 mg/mL, and 39.5% distilled water.
18. Oligolabeling buffer (OLB): 0.25 M Tris-HCl (pH 8.0), 25 mM MgCl₂, 0.36% (v/v) 2-mercaptoethanol, and 1 M hepes (pH 6.6).

2.4. Bacterial Strains and Plasmids

All plasmids used in this study were individually transformed into *E. coli* strain DH5 α (Invitrogen) for long-term storage and large-scale

amplification. The *pmi* gene of *E. coli* strain XL-1 Blue (Agilent Technologies) was PCR-amplified using gene-specific primers.

Four plasmids were used: plasmids pMI11 and pMI11G carrying the *pmi* gene alone or in combination with the β -glucuronidase (*gusA*) gene and driven by the maize ubiquitin promoter, respectively, and plasmids pMI3 and pMI3G carrying the *pmi* gene alone or in combination with the β -glucuronidase (*gusA*) gene and driven by the CaMV35S promoter, respectively.

3. Methods

3.1. DNA Delivery into Cultures

3.1.1. Large-Scale Plasmid Isolation

1. Inoculate the *E. coli* cultures containing either the pMI11, pMI11G, pMI3, or pMI3G plasmids (carrying *pmi* gene alone or together with *gusA* gene) from glycerol stock, streak on agar medium containing antibiotic (50 μ g/mL ampicillin), and incubate overnight at 37°C. Pick a single colony and inoculate into 10 mL LB medium containing antibiotic, and incubate by shaking overnight at 37°C (see Note 1). Then inoculate 1 mL of the culture into 500 mL of LB containing antibiotic and incubate by shaking overnight at 37°C.
2. Transfer the overnight culture into 250 mL centrifuge bottles and pellet the cells by centrifugation (3,000 $\times g$, 10 min and 4°C).
3. Carry out plasmid DNA isolation by using the QIAGEN Plasmid Maxi method (see Note 2). Subsequently, suspend the plasmid DNA obtained in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and store the DNA solution at a concentration of 1 μ g/ μ L at -20°C until needed. Analyze the DNA by electrophoresis on 1% agarose gels in TE buffer.

3.1.2. DNA-Microcarrier Preparation and Bombardment

1. Sterilize 60 mg gold microcarriers three times with 1 mL absolute ethanol, resuspend the final pellet in 1 mL of sterile distilled water, and keep as 50 μ L aliquots in microtubes, at 4°C (see Notes 3 and 4).
2. Add 5 μ L of DNA solution (1 μ g/ μ L), 50 μ L of CaCl₂ (2.5 M) and 20 μ L spermidine (0.1 M, free base form) into the 50 μ L gold suspension. Vortex the mixture for 3 min, spin for 10 s at 12,000 $\times g$, and discard the supernatant. Then wash the pellet with 250 μ L of absolute ethanol and finally resuspend in 60 μ L of absolute ethanol.
3. Load 6 μ L of the solution onto the centre of the macrocarrier and air-dry (see Note 5). Conduct bombardments at 1,100-psi rupture-disk pressure, 6-mm rupture disk to macrocarrier distance, 11-mm macrocarrier to stopping plate distance, 75-mm stopping plate to target tissue distance, and 67.5 mmHg vacuum pressure.

4. Incorporate two controls, i.e., tissues without bombardment and bombardment of microcarrier without DNA (see Note 6).
5. Incubate the bombarded tissues for 48 h at 28°C in the dark prior to transient GUS assay (15) overnight (20 h) at 37°C. Score the number of blue spots optically.
6. Consider each blue spot arising from the histochemical localization of GUS activity, whether in a single cell or a group of cells as a single transformation. These blue spots are an indicator of gene transfer into plant cells.

3.1.3. Optimal
Concentration of Selection
Agents

1. Embryogenic medium contains the following mixture of mannose and sucrose: 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, 30:0, 10:30, 20:30, and 0:0 g/L of mannose to sucrose. For each treatment, use three replicates and carry out twice.
2. In each plate, place 500 mg of embryogenic callus onto the media (incubate at 28°C; dark). After 30 days, transfer the embryogenic calli onto the same selection medium and incubate for another 30 days and repeat for four times. After 5 months, percentage of proliferation is measured using the formula modified from ref. 16.
3. In this experiment, consider the growth in 0:30 g/L of mannose to sucrose as 100% proliferation (control) assuming that there is no stress to reduce the proliferation rate. Divide the increase of calli weight on the selected media by the increased weight of control to give the percentage of proliferation relative to the control (Figs. 1 and 2):

$$\% \text{ of calli proliferation} = \frac{\text{Final weight} - \text{initial weight of selected tissues (g)}}{\text{Final weight} - \text{initial weight of control tissues (g)}} \times 100$$

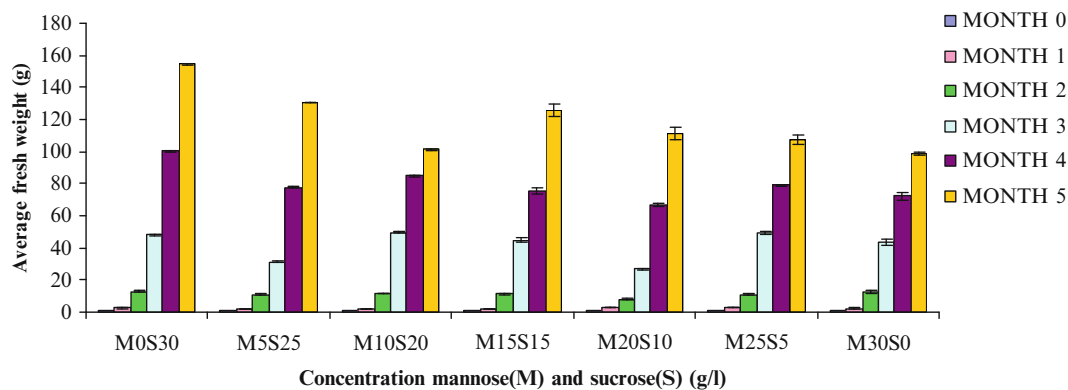


Fig. 1. Average fresh weight of nontransformed oil palm embryogenic calli in MS medium containing different combinations of mannose and sucrose concentrations for 5 months. Bars denote the standard error of the mean.



Fig. 2. Comparison of shoot proliferation on nontransformed oil palm embryogenic calli on media with or without mannose: (a) 0 g/L and (b) 30 g/L.

3.2. Regeneration of Transgenic Plants

3.2.1. Callus Initiation from Oil Palm Leaflet and Roots

Aseptically transfer either leaflets from an unopened (–6 position) frond or roots onto solid callus initiation medium and incubate at 28°C in the dark. Subculture the explants every 4 weeks onto the same medium until calli are formed (see Note 7).

3.2.2. Production of the Embryogenic Calli

Transfer the calli onto solidified embryogenic media and incubate at 28°C in the dark. Subculture the calli every 4 weeks onto the same medium and maintain until embryogenic calli are produced (see Note 8).

3.2.3. Transformation and Selection of Transformed Embryogenic Calli

1. Arrange the embryogenic calli as a circle (2-cm diameter) in the middle of a Petri dish containing embryogenic medium with osmoticum (0.4 M mannitol).
2. Bombard embryogenic calli on a Petri dish containing solidified embryogenic medium with 0.4 M mannitol. After 3 weeks, transfer the cultures onto solidified embryogenic media containing 30 g/L mannose.
3. After bombardment, transfer the embryogenic calli onto a fresh Petri dish containing embryogenic medium and incubate at 28°C in the dark for 3 weeks (see Note 9).
4. Subculture the calli every 4 weeks onto the same medium with selection agent until mannose-resistant embryogenic calli colonies are formed (see Note 10).
5. Once the resistant colonies reach the size of around 6–8 mm, isolate them and subject them to polyembryogenic calli formation (Fig. 3).



Fig. 3. Transformed oil palm embryoids on medium containing 30 g/L mannose.

3.2.4. Production of Polyembryogenic Cultures

Isolate resistant embryogenic callus colonies and transfer onto a fresh Petri dish containing solidified polyembryogenic media and incubate at 28°C in the presence of 16 h photoperiod (2,000 lx). Subculture the embryogenic callus colonies every 4 weeks onto the same medium with selection agent until polyembryogenic cultures are obtained (see Note 11).

3.2.5. Plantlet Regeneration

Transfer polyembryogenic calli to shoot induction medium (28°C: 2,000 lx). Subculture the calli every 4 weeks onto the same medium with selection agent until shoots are produced. Once the shoot is long enough, subject it to root induction.

3.2.6. Root Initiations

Transfer transformed shoots to liquid root induction medium in test tubes (28°C: 2,000 lx). Subsequently, transfer the small plantlets produced into soil in a biosafety screenhouse.

3.3. Molecular Analyses of Transgenic Plants

3.3.1. Preparation of Total DNA from Transformed Plants

1. Chill 1–10 g of leaves from transformed and untransformed plants, grind in liquid nitrogen prior to addition of 10-mL CTAB extraction buffer, and incubate at 65°C for 1 h (14). Remove protein and debris by 5 mL chloroform to isoamyl alcohol (24:1) extraction (4°C, 27,500 × *g* for 5 min). Transfer the aqueous phase into a new tube. This procedure is repeated twice.
2. Precipitate DNA by adding 6 mL chilled isopropanol (20 min; room temperature) and centrifuge at 27,500 × *g* for 5 min. Discard the supernatant and dry the pellet at room temperature.
3. Wash the pellet (washing buffer) at room temperature for 20 min before centrifugation at 27,500 × *g* for 5 min. Dry the final pellet, dissolve in TE buffer to a concentration of 1 µg/µL, and store at –20°C until needed.

3.3.2. Polymerase Chain Reaction

1. Amplify transgenes using standard and touchdown polymerase chain reaction (PCR) protocols (17). Carry out PCR in the PCR mixture. Use a standard PCR protocol involving 30 cycles: hot start 95°C (300 s), denature 92°C (50 s), annealing 60°C (50 s), elongation 72°C (60 s), and finally 120 s at 72°C (17) (see Note 12).
2. Check the amplified DNA fragments by electrophoresis on a 1.4% agarose gel in 1× TAE buffer (10 mM Tris, 5 mM Sodium Acetate, 0.5 mM EDTA, pH 8.0) (Fig. 4).

3.3.3. Southern Hybridization: Transfer of DNA from Agarose Gel to Nylon Membrane

1. Separate digested and undigested total DNA from transformed plants on 0.8% agarose gel in 1× TBE agarose gel. Transfer the DNA from agarose gel onto nylon membrane by capillary transfer (18).
2. Soak the agarose gel containing the digested (20 µg) and undigested (15 µg) DNA in depurinating buffer for 10 min, denature buffer for 45 min, and transfer into neutralization buffer for 1 h (18).
3. Transfer the gel onto 3MM paper with its ends soaked in 20× SSC buffer.
4. Place a piece of nylon membrane on top of the gel followed by two sheets of 3MM paper, paper towels, a glass plate, and a 500-g weight.
5. Leave the setup for 16–20 h to allow the entire DNA to transfer onto the membrane.
6. Wash the membrane with 2× SSC and UV cross-link.

3.3.4. Southern Hybridization: Preparation of Probes

1. Oligolabel the DNA fragments (~10 ng) to be used as probe (19) in 20 µL 5× OLB and 30% hexadeoxyribonucleotides (90 O.D. units/mL), boil for 5 min, then chill on ice (19).

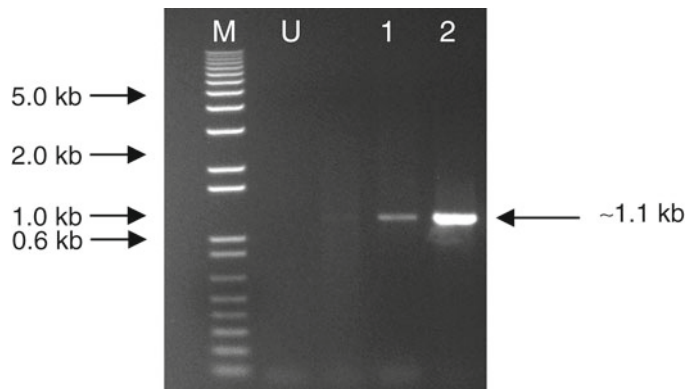


Fig. 4. PCR analysis of oil palm plantlets to amplify the *pmf* gene. The expected fragment size (1.1 kb) is indicated by the arrow. Lane M = 1 kb plus DNA ladder marker, U = untransformed negative control, and 1–2 = transformed oil palm.

2. Add the following to the above mixture: 2 μL 0.1 M dNTPs (except dCTP) (Amersham), 5 μL $\alpha^{32}\text{p(dCTP)}$ (370KBq/ μl) (Amersham), 2 μL 10 mg/mL BSA (Amersham), 1 μL klenow (6 U/ μL) (Amersham), and 14 μL distilled water. Incubate at 37°C for 30 min.
3. Denature the probe by the addition of 50 μL 1 M NaOH for 1 min, 50 μL 1 M HCl for 1 min, and 50 μL Tris-HCl (pH 7.5) for 1 min. Store on ice until use.

*3.3.5. Southern
Hybridization: Hybridization
Process*

1. Carry out prehybridization and hybridization using the same buffer.
2. Prehybridize the membrane with prehybridization buffer for 90 min at 65°C.
3. Add the denatured DNA probe and hybridize for 20 h at 65°C.
4. After hybridization, prewash the membrane for 1 min with 2 $\times$ SSC and wash twice in 0.1 SSC and 0.1% SDS: first for 30 min and the second for 45 min, both at 65°C. Wrap the washed membrane with saran wrap and expose to X-ray film with an intensifying screen at -70°C.

4. Notes

1. The use of a fresh bacterial colony from a glycerol stock has been proven to result in the highest plasmid DNA yield when using QIAGEN Plasmid Maxi kit. Even using a bacterial colony after 1 day storage at 4°C could result in up to 70% yield reduction.
2. Through experience, the QIAGEN Plasmid Maxi kit tubes could be reused for the same plasmid isolation up to four times without reduction in DNA yield. The tube should be rewashed and used as it is a new tube. Keeping the used tubes at 4°C for future use (maximum 1 week) is also recommended. However, reusing the tube for another plasmid is not recommended as contamination may occur. This is economical because the buffer used could be prepared in the lab as the formula is given in the brochure.
3. The use of gold microcarriers is better than tungsten because they are more spherical and uniform in size, biologically inert, nontoxic, and do not degrade DNA bonds. Tungsten, on the other hand, is highly heterogeneous in both size and shape, potentially toxic to some cell types, and subjected to surface oxidation. It has also been reported to acidify and degrade DNA bonds (20) and in comparison to gold, to have a greater tendency to aggregate during precipitation (21).

4. Of the different brands of microfuge tubes evaluated for biolistic transformation, the Treff microtube has been reported to be the best (20), and this was utilized in all oil palm transformation experiments.
5. Only 6 μ l of DNA-gold solution are loaded onto the center of the macrocarrier; additional loading will be wasted as it will not pass through the stopping screen area. Furthermore, when aliquoting the DNA-gold solution from the microfuge tube, make sure the tube is immediately closed after use as the ethanol will evaporate. Thus, a smaller number of bombardments could be carried out as well as higher amounts of DNA-gold will be delivered which could cause damage to the receiving target tissue.
6. It is important to have at least two controls for bombardment to ensure final interpretation of results will not be questionable. Tissue without bombardment is used as a negative control for the transformation. Bombardment without DNA is to ensure all the materials used, (gold microcarrier, spermidine, CaCl_2 , and water) are not contaminated with any DNA source.
7. Explants are handled in a laminar flow cabinet to ensure its sterility. Oil palm callus is normally obtained after a minimum period of 3 months. Sometimes, it may take a longer time, i.e., up to 12 months. After 12 months, the explants which fail to produce callus are discarded. It must be highlighted here that the oil palm tissue culture process is very highly genotype dependent. Not all the explants cultured will eventually produce callus.
8. Embryogenic calli are normally produced from callus around 3–6 months after culturing. Some cultures may take a shorter time and some may take longer. Not all calli will become embryogenic. Normally less than 50% of the calli will eventually produce embryogenic calli. The chance of embryogenic calli to produce polyembryogenic calli is high.
9. After bombardment and before starting the selection, always use fresh media for maintaining the nutrient supply for helping in efficient selection and recovery of transformants. After subculturing onto new plates for selection and proliferation, always inspect the plates regularly for signs of contamination. Discard any contaminated cultures to avoid or minimize cross contamination to other cultures in the incubator.
10. Selections are normally carried out 3 weeks postbombardment to ensure that the bombarded embryogenic calli have recovered from stress and doubled at least once in order to be ready for exposure to selection agent. Selection is a slow process and quite discouraging, especially when the cultures turn dark brown in medium containing mannose. Normally, these resistant

embryogenic callus colonies will appear at least 5–6 months after exposure to the selection agent.

11. Once the resistant embryogenic callus colony reaches the size of around 6–8 mm, it will be individually isolated by removing the surrounding dead cultures and transferring onto polyembryogenic inducing medium. The size is important to ensure that a culture is able to proliferate and regenerate by itself. Using smaller size of calli will cause most of the calli to lose its regeneration capability and eventually die. This part of the selection process is critical.
12. Initially, PCR is carried out using internal control primers for oil palm which will specifically amplify a 1-kb fragment of the oil palm genome. All samples (including untransformed control) are subjected to amplification using the internal control primers. In the event that the control band is not observed, the DNA sample is further purified until the band is produced. The internal control is important to ensure that untransformed samples have failed to amplify transgenes for reasons other than low quality or quantity of host genomic DNA. Later, conduct the same PCR for the selectable marker gene, *pmi*.

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Chapter 15

Transformation of Oil Palm Using *Agrobacterium tumefaciens*

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and Mat Yunus Abdul Masani

Abstract

Transgenic oil palm (*Elaeis guineensis* Jacq.) plantlets are regenerated after *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli derived from young leaves of oil palm. The calli are transformed with an *Agrobacterium* strain, LBA4404, harboring the plasmid pUBA, which carries a selectable marker gene (*bar*) for resistance to the herbicide Basta and is driven by a maize ubiquitin promoter. Modifications of the transformation method, treatment of the target tissues using acetosyringone, exposure to a plasmolysis medium, and physical injury via biolistics are applied. The main reasons for such modifications are to activate the bacterial virulence system and, subsequently, to increase the transformation efficiency. Transgenic oil palm cells are selected and regenerated on a medium containing herbicide Basta. Molecular analyses revealed the presence and integration of the introduced *bar* gene into the genome of the transformants.

Key words: Transgenic oil palm, *Agrobacterium*-mediated, *bar* gene, Herbicide Basta™

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is the most important economic crop for Malaysia. The Malaysian oil palm industry has become one of the most highly organized sectors of any national agricultural system in the world. It is able to compete with other vegetable oil products from developed and developing countries. The total exports of oil palm products, consisting of palm oil, palm kernel oil, palm kernel cake, oleochemicals, biodiesel, and finished products, increased by 2.8% or 0.63 million tonnes to 23.06 million tonnes in 2010 from 22.43 million tonnes, as recorded in 2009 (1). In order for oil palm to maintain its premier position and to remain competitive, increasing yield per unit area as well as producing novel high-value products using approaches such as genetic engineering is essential (2).

To genetically engineer oil palm for modifying fatty acid composition or producing novel products, establishment of a reliable transformation method is essential.

Agrobacterium tumefaciens is one of nature's most successful plant genetic engineers and is routinely used to transfer desirable genes into dicotyledonous plants. *Agrobacterium*-mediated transformation is relatively efficient and leads to a low copy number of intact and nonrearranged transgenes, frequently integrated into the plant genome (3). The concept of using *Agrobacterium* as a tool to create transgenic plants is promising for monocotyledonous plants even though it was at one time thought to be an obstacle because monocots are not the natural host for *Agrobacterium* (4). The first transgenic monocot plant mediated by *Agrobacterium* was *Asparagus officinalis* (5). This was achieved by inoculating asparagus calli with *Agrobacterium* containing a Ti plasmid carrying *neomycin phosphotransferase* (*npt II*) gene in the T-DNA region as a selectable marker. As for commercial crops, rice (6) and maize (7) were the first monocotyledonous plants transformed with *Agrobacterium*. Following these achievements, transgenic plants obtained *via Agrobacterium*-mediated transformation have been regenerated in more than a dozen monocotyledonous species, ranging from the most important cereal crops to ornamental plant species. Efficient transformation protocols for agronomically important cereal crops such as rice, wheat, maize, barley, and sorghum have been developed, and transformation for some of these species has become routine (8).

The above successes make it possible to apply the *Agrobacterium*-mediated method to transform monocot plants such as oil palm. Studies are ongoing to modify this monocotyledonous plant through genetic engineering to improve the quality of the palm oil (9). Transgenic oil palm has been produced previously using the biolistic method (2). However, this transformation method tends to introduce multiple copies of the transgenes which could lead to gene silencing. The *A. tumefaciens* method, on the other hand, has been proven to introduce single or low copies of transgenes (10). Thus, the protocols for *A. tumefaciens*-mediated transformation of oil palm embryogenic calli and selection of transformed cells, as well as the protocols for regeneration transgenic oil palm, and finally the integration and expression of transgenes in the regenerated transgenic oil palm are described below.

2. Materials

2.1. Equipment

Gene Pulser (Bio-rad), electroporation cuvette (Bio-Rad), biolistics—PDS-1000/He Apparatus (Bio-Rad), autoclave (Tomy), incubator shaker (Innova), electrophoresis unit and power

supplies (Bio-Rad), PCR apparatus (MJ Research—PTC-100 and PTC-200), centrifuge RC5C *Plus* (Sorvall), UV cross-linked (XL-1500 UV Crosslinker), hybridization oven (Techne Hybridiser HB-1D), vortex (Finemixer Sh2000, FinePCR), pH meter (Mettler Toledo), hot plate and stirrer, analytical balance, plant tissue-culture incubator (Convion TC-16), and microcentrifuge (Hettich Mikro120).

2.2. Supplies

Gold microcarriers (Bio-Rad), macrocarrier (Bio-Rad), stopping screen (Bio-Rad), rupture disk (Bio-Rad), Treff microtube (Switzerland), Petri dish, nylon membrane (Hybond-N+, Amersham), 3MM paper (Whatman), and X-ray film (Kodak). Chemicals from Sigma unless stated otherwise.

2.3. Bacterial Strain and Plasmids

Agrobacterium tumefaciens, LBA 4404, contains pCambia1300-Uba (containing Basta-resistant gene driven by the maize ubiquitin promoter).

2.4. Media and Solutions

1. Bacterial LB medium: 10 g NaCl, 10 g tryptone (Pronadisa), and 5 g yeast extract (Pronadisa), pH 7.0 in 1 L distilled water.
2. Bacterial induction medium: MS macro- and micronutrients (11) and Y3 vitamins (12) (1 mg/mL thiamine-HCl, 1 mg/mL pyridoxine HCl, 1 mg/mL nicotinic acid) supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-asparagine, 5 μ M NAA, and 60 g/L sucrose. The medium is adjusted to pH 5.7 with KOH prior to autoclaving. The medium is supplemented with filter-sterilized 200 μ M acetosyringone (Duchefa) after autoclaving. Acetosyringone is prepared as a stock solution of 0.2 M in DMSO.
3. Cocultivation medium: MS macro- and micronutrients (11) and Y3 vitamins (12) supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-asparagine, 5 μ M NAA, 0.7% agar, and 30 g/L sucrose, pH 5.7, in the presence of 200 μ M acetosyringone.
4. Callus initiation medium: MS salts (11), Y3 vitamins (12) (1 mg/mL thiamine-HCl, 1 mg/mL pyridoxine-HCl, 1 mg/mL nicotinic acid), 0.1 mg/mL *myo*-Inositol, 0.1 mg/mL L-glutamine, 3% sucrose, 5×10^{-5} M, 2,4-D, 0.25% activated charcoal, and 0.7% agar, pH 5.7 (13).
5. Embryogenic callus medium: MS macro- and micronutrients (11) and Y3 vitamins (12) (1 mg/mL thiamine-HCl, 1 mg/mL pyridoxine HCl, and 1 mg/mL nicotinic acid), 100 mg/L each of *myo*-inositol, L-glutamine, L-asparagine, 10 μ M 2,4-D, 5 μ M NAA, 30 g/L sucrose, and 0.8% agar, pH 5.7 (13).
6. Plasmolysis medium: MS macro- and micronutrients (11), Y3 vitamins (12) supplemented with 100 mg/L each of

myo-inositol, L-glutamine, L-asparagine, 5 μ M NAA, and 60 g/L sucrose, pH 5.7 (13).

7. Polyembryogenic culture medium: MS macro- and micronutrients (11) and Y3 vitamins (12) supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-arginine and L-asparagine, 5 μ M IBA, 0.7% agar, and 30 g/L sucrose (13).
8. Shoot induction medium: MS macro- and micronutrients, (11) Y₃ vitamins (12) supplemented with 100 mg/L each of *myo*-inositol, L-glutamine, L-arginine and L-asparagine, 0.1 μ M NAA, 0.4% agar, and 30 g/L sucrose. Adjust the medium to pH 5.7 with KOH prior to autoclaving (13).
9. Liquid root induction medium: MS macro- and micronutrients (11) and Y3 vitamins (12) supplemented with 300 mg/L L-glutamine, 100 mg/L *myo*-inositol, 10 μ M 2,4-D, 70 μ M NAA, 0.15% activated charcoal, and sucrose at 60 g/L. Adjust the medium to pH 5.7 with KOH prior to autoclaving (13).
10. PCR mixture: DNA (50 ng total DNA or 5 ng plasmid DNA), reaction buffer (50 mM KCL and 10 mM Tris-Cl pH 8.3) (Promega), dNTPs (200 μ M each) (Promega), 1.5 mM MgCl₂ (Promega), primers, and Taq DNA polymerase (Perkin-Elmer).
11. CTAB buffer: 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 NaCl, 2% CTAB, 1% PVP, and 0.2% 2-mercaptoethanol (23).
12. Washing buffer: 76% ethanol and 1 mM ammonium acetate.
13. TE buffer: 10 mM Tris-HCl pH 7.5 (10 mM Tris) and 1 mM EDTA.
14. TBE buffer: 45 mM Tris-Borate and 1 mM EDTA, pH 8.0.
15. Depurinating buffer: 0.2 M HCl.
16. Denaturing buffer: 1.5 M NaCl and 0.5 M NaOH.
17. Neutralization buffer: 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl.
18. SSC buffer: 175.3 g NaCl and 88.2 g sodium citrate in 1 L, pH 7.0.
19. Prehybridization buffer: 40% pipes/NaCl pH 6.8 (1.5% pipes, 8.7% NaCl and 0.37% EDTA, pH 8.0), 20% Denhardtts 50 $\times$ (1% BSA, 1% Ficoll, 1% PVP, and 10% SDS) (Promega), 0.5% ssDNA 10 mg/mL, and 39.5% distilled water (14).
20. Oligolabeling buffer (OLB): 0.25 M Tris-HCL (pH 8.0), 25 mM MgCl₂, 0.36% (v/v) 2-mercaptoethanol, and 1 M hepes (pH 6.6) (15).

3. Methods

1. Streak *A. tumefaciens*, LBA 4404 on an LB plate containing filter-sterilized 50- $\mu\text{g}/\text{mL}$ kanamycin, 20- $\mu\text{g}/\text{mL}$ rifampicin, and 100- $\mu\text{g}/\text{mL}$ streptomycin sulfate after autoclaving and cooling down to 55°C. Incubate for 2 days at 27°C.
2. Transfer a single colony into a 50-mL centrifuge tube containing 20 mL LB and incubate on a rotary shaker for 16 h.
3. Transfer 1 mL of the inoculum into fresh 20 mL LB and further incubate overnight.
4. Spin the overnight culture at $4000 \times g$ for 15 min, resuspend the pellet using 25 mL bacterial induction medium, and then dilute until it reaches an optical density of 0.2 at 600 nm (see Note 1).

3.1. Transformation of Embryogenic Calli

Transfer the embryogenic calli obtained from suspension cultures (Fig. 1a) onto embryogenic callus medium and incubate at 28°C in the dark for 1 day prior to transformation (see Note 2).

3.1.1. Embryogenic Calli

3.1.2. Pretreatment of Embryogenic Calli

1. Plasmolyze the embryogenic calli in 50 mL plasmolysis medium in the presence of 200 μM acetosyringone by shaking (100 rpm) at 28°C in the dark for 1 h prior to bombardment (see Note 3).
2. Collect the calli by discarding the plasmolysis medium and arrange as a circle in the middle of a Petri dish containing embryogenic medium for bombardment using the method described (2) (see Note 4).
3. Sterilize 60 mg gold microcarriers three times with 1 mL absolute ethanol and resuspend the final pellet in 1 mL of sterile distilled water and keep as 50 μL aliquots in microtubes, at 4°C.
4. Add 50 μL of CaCl_2 (2.5 M), and 20 μL spermidine (0.1 M, free base form) into the 50 μL gold suspension. Vortex the mixture for 3 min, spin for 10 s at $12,000 \times g$, and discard the supernatant. Then wash the pellet with 250 μL of absolute ethanol and finally resuspend in 60 μL of absolute ethanol.
5. Load 6 μL of the solution onto the center of the macrocarrier and air dry. Conduct bombardments at 1,100-psi rupture-disk pressure, 6-mm rupture disk to macrocarrier distance, 11-mm macrocarrier to stopping plate distance, 75-mm stopping plate to target tissue distance, and 67.5 mmHg vacuum pressure.

3.1.3. Agrobacterium-Mediated Transformation of Embryogenic Calli

1. Transfer the bombarded calli into flasks containing an *Agrobacterium* suspension and shake slowly (80 rpm) for 2 h at 27°C in the dark. Then, posttreat the calli by blotting dry on

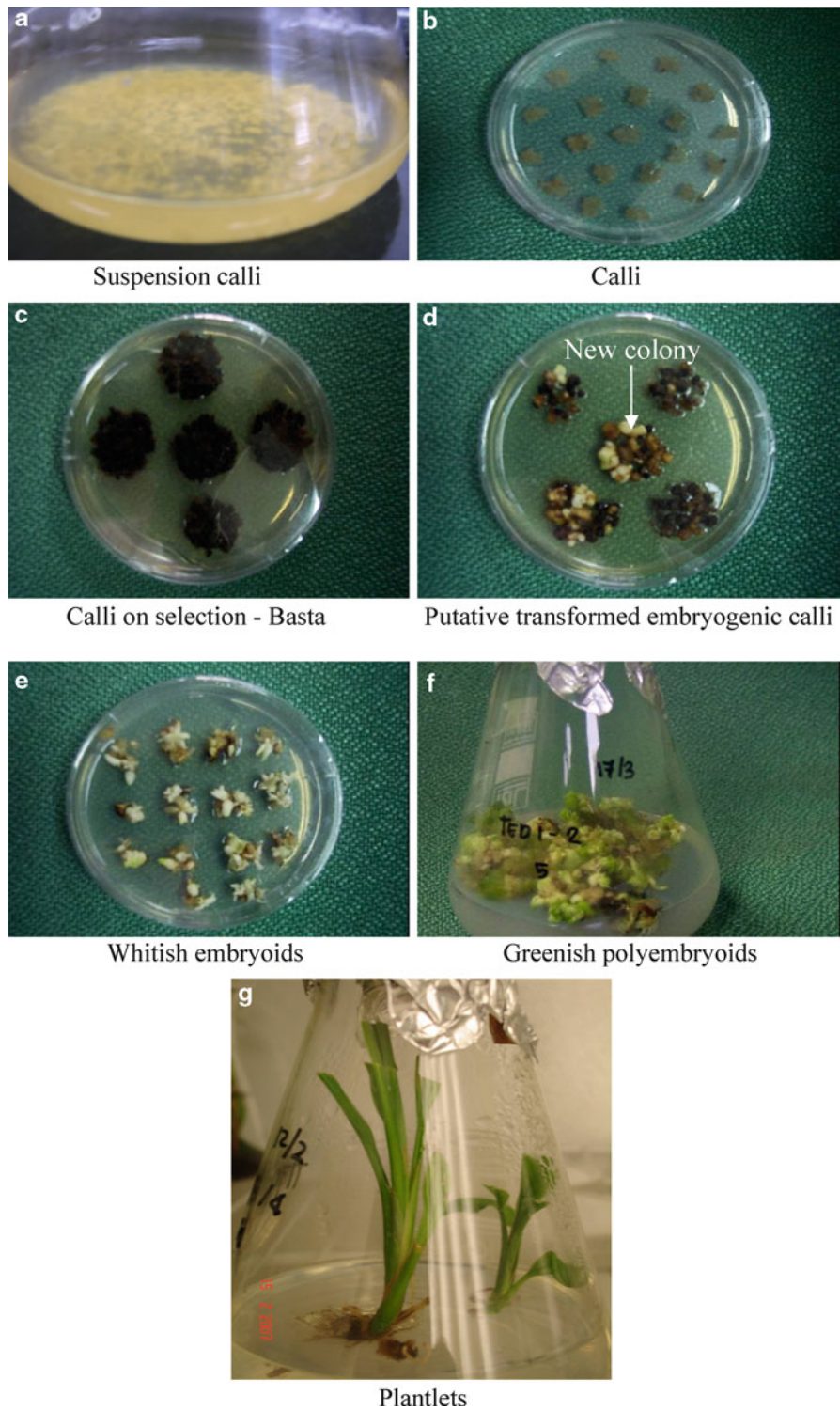


Fig. 1. Development of transgenic oil palm.

sterile filter paper and transfer onto the plasmolysis medium for 1 day at 28°C in the dark (see Note 5).

2. Transfer the calli onto cocultivation medium and culture for 3 days at 28°C in the dark.
3. Wash the calli several times using liquid embryogenic callus medium with addition of timentin (100 µg/mL) and a plant preservative mixture (0.2%) to kill the bacteria (see Note 6).
4. Blot dry the calli and culture on the embryogenic callus medium containing timentin (50 µg/mL) and plant preservative mixture (0.1% w/v) for a month (Fig. 1b).
5. Transfer the calli onto a selection medium (embryogenic callus medium) containing 50 µg/mL of the herbicide Basta (Fig. 1c) and subculture every 4 weeks onto the same medium until Basta-resistant embryogenic calli colonies are formed (Fig. 1d) (see Note 7).
6. Isolate Basta-resistant embryogenic calli colonies, transfer onto a fresh Petri dish containing solidified polyembryogenic medium, and incubate at 28°C in the presence of light (Fig. 1e). Subculture the embryogenic calli colonies every 4 weeks onto the same medium with selection agent until polyembryogenic cultures are obtained (Fig. 1f) (see Note 8).
7. Transfer polyembryogenic calli to shoot induction medium (28°C: 2,000 lx). Subculture the calli every 4 weeks onto the same medium with selection agent until shoots are produced (Fig. 1g). Once the shoot is long enough, subject it to root induction (see Note 9).
8. Transfer transformed shoots to liquid root induction medium in test tubes (28°C: 2,000 lx). Subsequently, transfer small plantlets into soil in a biosafety greenhouse.

3.2. Transgenic Analysis

3.2.1. Plant DNA Extraction

1. Chill 1–10 g of leaves from transformed and untransformed plants and grind in liquid nitrogen prior to addition of 10 mL CTAB extraction buffer and incubate at 65°C for 1 h (23).
2. Remove protein and debris by 5 mL chloroform to isoamyl alcohol (24:1) extraction (4°C, 27,500 × *g* for 5 min).
3. Transfer the aqueous phase into a new tube. This procedure is repeated twice.
4. Precipitate DNA by adding 6 mL chilled isopropanol (20 min; room temperature) and centrifuge at 27,500 × *g* for 5 min. Discard the supernatant and dry the pellet at room temperature.
5. Wash the pellet with washing buffer at room temperature for 20 min before centrifugation at 27,500 × *g* for 5 min. Dry the final pellet and dissolve in TE buffer to a concentration of 1 µg/µl and store at –20°C until needed.

3.2.2. Polymerase
Chain Reaction

1. Amplify transgenes using standard and touchdown polymerase chain reaction (PCR) protocols. Carry out PCR reactions in the PCR mixture. Use a standard PCR protocol involving 30 cycles: hot start 95°C (300 s), denature 92°C (50 s), annealing 60°C (50 s), elongation 72°C (60 s), and finally 120 s at 72°C (17). Use the following touchdown procedure: hot start at 95°C (300 s), denature at 94°C (45 s), annealing at 70°C (45 s; -0.5°C per cycle for 10 cycles), and elongation at 72°C (60 s) followed by 24 cycle of 94°C (45 s), 65°C (45 s), 72°C (60 s), and finally 72°C for 120 s (see Note 10).
2. Check the amplified fragments using agarose gel electrophoresis on a 1.4% agarose gel in 0.5× TBE buffer (Fig. 2).

3.2.3. Southern
Hybridization: Transfer
of DNA from Agarose
Gel to Nylon Membrane

1. Separate the undigested total DNA from transformed plants on 0.8% agarose gel in 1× TBE agarose gel. Transfer the DNA from agarose gel by capillary transfer onto a nylon membrane (14).
2. Soak the agarose gel containing the undigested (100 µg) DNA in depurinating buffer for 15 min, denature buffer for 20 min, and transfer into neutralization buffer for 20 min.
3. Transfer the gel onto 3MM paper with its end soaked in 20× SSC buffer.
4. Place a nylon membrane on top of the gel followed by two sheets of 3MM paper, paper towels, a glass plate, and a 500-g weight.
5. Leave the setup for 16–20 h to allow the entire DNA to transfer onto the membrane.
6. After transfer, wash the membrane with 2× SSC and UV cross-link.

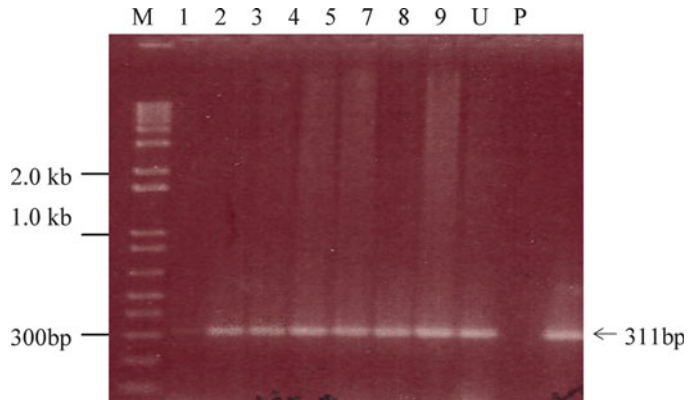


Fig. 2. Polymerase chain reaction (PCR) analysis on genomic DNA of oil palm leaves with *bar* gene-specific primers. Lane M: 1 kb Plus DNA ladder; lane P: plasmid pUBA; lanes 1–9: DNA samples; and lane U: untransformed plant. Arrow indicates PCR products of 311 bp.

3.2.4. Southern

Hybridization:

Preparation of Probes

1. Oligolabel the DNA fragments (~10 ng) to be used as probe (15) in 20 μL 5 $\times$ OLB and 30% hexadeoxyribonucleotides (90 O.D. units/mL), boil for 5 min, then chill on ice (15).
2. Add the following to the above mixture: 2 μL 0.1 M dNTPs (except dCTP) (Amersham), 5 μL $\alpha^{32}\text{p}$ (dCTP) (370KBq/ μL) (Amersham), 2 μL 10 mg/mL BSA (Amersham), 1 μL klenow (6 U/ μL) (Amersham), and 14 μL distilled water. Incubate at 37°C for 30 min.
3. Denature the probe by the addition of 50 μL 1 M NaOH for 1 min, 50 μL 1 M HCl for 1 min, and 50 μL Tris-HCl (pH 7.5) for 1 min. Store on ice until use.

3.2.5. Southern

Hybridization: Hybridization

1. Carry out prehybridization and hybridization using the same buffer.
2. Prehybridize the membrane with prehybridization buffer for 90 min at 65°C.
3. Add the denatured DNA probe and hybridize for 20 h at 65°C.
4. After hybridization, prewash the membrane for 1 min with 2 $\times$ SSC and wash twice in 0.1 SSC and 0.1% SDS: first for 30 min and the second for 45 min, both at 65°C.
5. Wrap the washed membrane with saran wrap and expose to X-ray film with an intensifying screen at -70°C (Fig. 3).

3.2.6. Analysis of Herbicide

Basta Resistance

1. Paint leaves from transformed and untransformed plants with full strength Basta solution containing 01% Tween 20 (16) to approximately two-thirds of each leaf.
2. Evaluate symptoms 2 weeks after application (see Note 11).

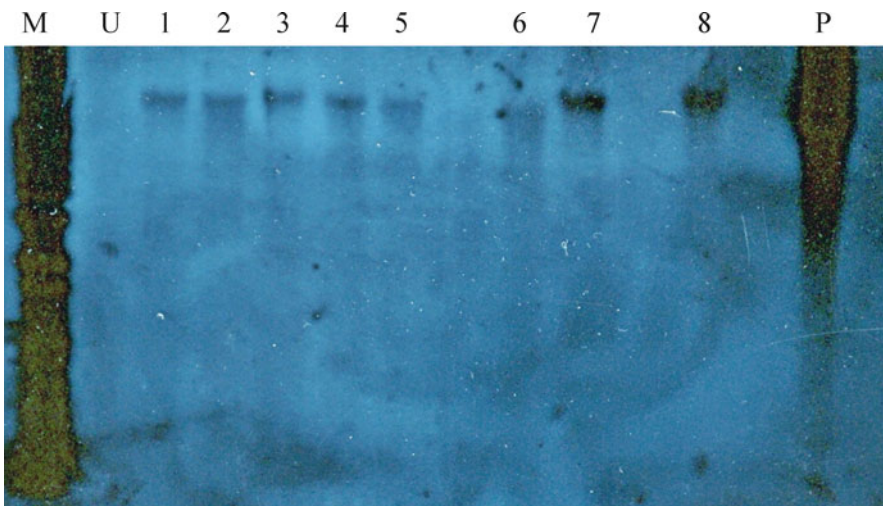


Fig. 3. Southern blot analysis of undigested genomic DNA of transgenic oil palm plants. Lane M: 1 kb Plus DNA ladder; Lane U: untransformed plant, Lane P: plasmid pUBA; Lanes 1–8: samples of transgenic oil palm.

4. Notes

1. Through experience, a higher density ($>OD_{600}=0.2$) of *Agrobacterium* suspension will result in difficulty in eliminating the bacterial cells even using concentrations of timentin higher than 100 $\mu\text{g}/\text{mL}$ and plant preservative mixture higher than 0.2% v/v.
2. Embryogenic suspension calli are chosen as the starting material for transformation because they can be easily regenerated. The use of highly regenerative tissue as a transformation target tissue often results in the regeneration of a large number of independently transformed lines as observed in several monocot crop species such as rice (18) and wheat (19).
3. Pretreatment of the embryogenic calli onto plasmolysis medium which contained acetosyringone will help to induce the *vir* gene transfer (20). In addition, plasmolysis of the embryogenic calli will minimize the bombardment damage and permit the maximum embryogenic calli response (21).
4. The Biolistic approach to create wounding was carried out to promote the transformation process. Biolistic bombardment has been shown to be an efficient tool for wounding plant tissues prior to *Agrobacterium* transformation (22).
5. Posttreatment of the embryogenic calli onto plasmolysis medium after bombardment is intended to facilitate stabilization of cell membranes for faster healing of the lesion caused by microcarrier penetration and reduce turgor pressure of cells to prevent leakage and cell rupture.
6. The transformed embryogenic calli were cultured on a medium containing timentin and plant preservative mixture for a month to ensure that the *Agrobacterium* are completely killed. In addition, the medium without the selective agent are also to promote the growth of calli before exposing to selection agent.
7. Upon transferring to a medium containing Basta, untransformed cells began to die, allowing only resistant cells to proliferate. Normally, Basta-resistant embryogenic calli (a new colony) appeared after 5–6 months in the selection medium.
8. The Basta-resistant embryogenic calli were continuously subcultured onto fresh selection medium for proliferation and regeneration. The size of the colonies became bigger and turned into embryoids. The transgenic embryoids began to regenerate into whitish embryoids followed by greenish polyembryogenic calli after 3–5 months of culture on the selection medium.

9. After 2–3 months, shoots appeared from the polyembryogenic calli. The shoots were individually isolated and cultured in flasks containing the shoot induction medium for shoot elongation. After 2–3 months, the elongated shoots were transferred into test tubes containing root inducing medium for root initiation and further development. It was observed that the selection and regeneration process for the production of transgenic oil palm using *Agrobacterium*-mediated transformation is similar to the production of transgenic oil palm using the microprojectile bombardment method (2).
10. Touchdown PCR procedure is used for the Basta-resistant gene because the gene has a high GC content (more than 60%). The use of normal PCR protocols normally resulted in the amplification of nonspecific and false positive bands. The use of touch down is very successful in specifically amplifying the Basta-resistant gene.
11. One of the effective and easiest approaches to test for Basta resistance in transformed oil palm plants is by direct painting of leaves with an herbicide solution. The leaves from control palms became brown and wilted after 2 weeks, compared to the transformed plants which remained healthy and green. These results showed that the transformed plants are resistant to Basta, and that the transgene is stably expressed.

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Part V

Transformation Technology: Dicots

Chapter 16

Highly Efficient Transformation Protocol for Plum (*Prunus domestica* L.)

César Petri, Ralph Scorza, and Chinnathambi Srinivasan

Abstract

A high-throughput transformation system for plum has been developed using hypocotyl slices excised from zygotic embryos as the source of explants. The hypocotyl slices are infected in an *Agrobacterium tumefaciens* suspension and then cocultivated for 3 days in shoot regeneration $\frac{3}{4}$ MS basal medium supplemented with 9 μ M 2,4-dichlorophenoxyacetic acid. Transgenic shoots are regenerated in a medium containing 7.5 μ M thidiazuron and elongated in a medium containing 3 μ M benzyladenine in the presence of 80 mg/L kanamycin in both media. Transformed shoots are rooted in $\frac{1}{2}$ MS basal medium supplemented with 5 μ M NAA and 40 mg/L kanamycin. The transgenic plants are acclimatized in a growth chamber and transferred to a temperature-controlled greenhouse. This protocol has allowed transformation efficiencies up to 42% and enabled the production of self-rooted transgenic plum plants within 6 months of transformation.

Key words: High-throughput transformation, *Prunus domestica*, Fruit trees, Rosaceae, Functional genomics, *Agrobacterium tumefaciens*

1. Introduction

Fruit trees are among the most recalcitrant of plants to regenerate adventitious shoots from cultured explants. In most woody fruit species, transformation and regeneration are generally difficult and often limited to a few genotypes or to seedlings (1). This feature is the major limiting factor preventing the development of gene transfer technologies for fruit trees (2). Plum has been one of the more successful rosaceous fruits to regenerate and transform, especially among *Prunus* species. Although several regeneration protocols have been reported from different tissues of European plum (*Prunus domestica* L.), there are few reports of regeneration of transformed shoots (3).

Mante et al. (4) were the first to develop an *Agrobacterium*-mediated transformation protocol in plum using embryonic hypocotyl slices as the explant source. This protocol was enhanced by optimizing the selection [80 mg/L kanamycin (km) was added just after cocultivation] as well as rooting and acclimatization steps and reached 4.2% transformation efficiency (5). Recently, the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) to cocultivation medium and the optimization of the timing of each step in the protocol have improved the efficiency of transformation up to 42% (6).

Since 1991, this method, in its different updates, has been employed successfully for the introduction of agronomically useful genes into this species (5–12). The high efficiency of the current system, coupled with the rapid plant establishment methodology, would also enable functional genomics studies (6).

We show that the plum transformation protocol is a simple, reproducible, and highly productive system for developing transgenic plum lines, making possible the use of this species as a model plant for rosaceous fruit trees specifically and woody plants in general.

2. Materials

2.1. Plant Material: Collection and Preparation

1. Seeds from the plum cultivar “Bluebyrd” stored at 4°C (see Note 1).
2. Commercial bleach.
3. Tween-20 (Sigma-Aldrich).

2.2. *Agrobacterium* Strain, Plasmid, Bacterium Culture, and Tissue Culture Medium

1. Stock solutions:
 - (a) 0.1 mM acetosyringone (AS): Dissolve 98.1 mg AS in 5 mL dimethyl sulfoxide (DMSO) and store at 4°C for 4 weeks.
 - (b) 20 mg/mL gentamicin sulfate: Dissolve in water, filter sterilize, and store at –20°C in aliquots.
 - (c) 50 mg/mL kanamycin sulfate: Dissolve in water, filter sterilize, and store at –20°C in aliquots.
 - (d) 10× Murashige and Skoog (MS) Basal salt macronutrient (13) (liquid, Sigma-Aldrich). Store at 4°C.
 - (e) 10× MS Basal salt micronutrient (13) (liquid, Sigma-Aldrich). Store at 4°C.
2. *Agrobacterium tumefaciens* strain GV3101 (derivative of C58) harboring the pBINPLUS/ARS binary plasmid (14). This plasmid contains *nptII* as a selectable marker gene, conferring aminoglycoside antibiotics resistance to transformed cells.

3. Liquid Luria-Bertani medium (LB): Dissolve 10 g/L Bacto-tryptone (Difco Laboratories, Detroit, MI, USA), 5 g/L Bacto-yeast extract (Difco Laboratories), and 10 g/L NaCl in 800 mL ddH₂O. Adjust pH to 7.0 with KOH, and make up to 1 L final volume with ddH₂O and sterilize by autoclaving. Store at room temperature and add antibiotics as necessary to culture *Agrobacterium* strains containing plasmids of interest.
4. Solid LB medium: Add 15 g/L Bacto agar to LB. Cool down the medium to 45–50°C. Add antibiotics as necessary.
5. Bacterial resuspension medium (BRM): Full MS salts (macro- and micronutrients), 2% (w/v) sucrose (pH 5.8), and 100 µM AS. Mix MS salts and sucrose and adjust pH to 5.8 with KOH. Sterilize the resuspension medium by autoclaving. Cool down the medium to 45–50°C. Add AS after autoclaving.

2.3. Co-cultivation

1. Stocks:
 - (a) 1,000× MS vitamins (12) (powder, Sigma-Aldrich). Dissolve in distilled water, filter sterilize, make 1-mL aliquots in 1.5-mL microfuge tubes, and store at –20°C.
 - (b) 75 mM thidiazuron (TDZ): Dissolve 16.5 mg TDZ in 1 mL DMSO. Store at 4°C for 4 weeks.
 - (c) 1 mM 3-indolbutyric acid (IBA). Dissolve 20.3 mg IBA in 1 mL KOH 1 N. Add distilled water up to 100-mL final volume. Store at 4°C.
 - (d) 9 mM 2,4-dichlorophenoxyacetic acid (2,4-D). Dissolve 20 mg 2,4-D in 1 mL 96% ethanol. Store at 4°C.
2. Shoot Regeneration Medium (SRM) supplemented with 9 µM 2,4-D (SRMD): Mix $\frac{3}{4}$ MS macronutrients, $\frac{3}{4}$ MS micronutrients, full MS vitamins, 2% (w/v) sucrose, 7.5 µM TDZ, 0.25 µM IBA, and 9 µM 2,4-D. Adjust pH to 5.8 with KOH, add 0.7% (w/v) Bacto agar (Difco Laboratories), and sterilize SRMD by autoclaving (see Note 2).

2.4. Transgenic Shoot Regeneration

1. SRM (without 2,4-D) supplemented with 80 mg/L kanamycin and 300 mg/L timentin (see Note 3): Mix $\frac{3}{4}$ MS macronutrients, $\frac{3}{4}$ MS micronutrients, full MS vitamins, 2% (w/v) sucrose, 7.5 µM TDZ, and 0.25 µM IBA. Adjust pH to 5.8 with KOH and add 0.7% (w/v) Bacto agar (Difco Laboratories). Autoclave to sterilize. Cool down the medium to 45–50°C. Dissolve the antibiotics (powder) in 10 mL distilled water, filter sterilize with 0.2-µm syringe filter, and add them to the medium.
2. Bacterial washing liquid medium: Mix $\frac{1}{2}$ MS macronutrients and micronutrients and adjust pH to 5.8 with KOH. Autoclave to sterilize. Cool down the medium to 45–50°C and add filter-sterilized timentin (300 mg/L).

2.5. Shoot Elongation and Multiplication

1. Stock solution:
 - (a) 1 mM 6-benzylaminopurine (BA). Dissolve 22.5 mg BA in 1 mL KOH 1 N. Add distilled water to 100 mL final volume. Store at 4°C up to 4 weeks (see Note 4).
2. Shoot-growing medium (SGM) supplemented with 80 mg/L kanamycin and 300 mg/L timentin: $\frac{3}{4}$ MS macronutrients, $\frac{3}{4}$ MS micronutrients, full MS vitamins, 2% (w/v) sucrose, 3.0 μ M BA, and 0.25 μ M IBA. Adjust pH to 5.8 with KOH and add 0.7% (w/v) Bacto agar (Difco Laboratories, Detroit, MI, USA). Autoclave to sterilize. Cool down the medium to 45–50°C. Dissolve the antibiotics (powder) in 10 mL water, filter sterilize with 0.2- μ m filter, and add them to SGM medium.

2.6. Rooting

1. Stocks:
 - (a) 1 mM α -naphthaleneacetic acid (NAA). Dissolve 18.6 mg NAA in 1 mL KOH 1 N. Add distilled water up to 100 mL final volume. Store at 4°C.
 - (b) 1 mM kinetin (Kin). Dissolve 21.5 mg Kin in 1 mL KOH 1 N. Add distilled water up to 100 mL final volume. Store at –20°C.
2. Rooting medium (RM) supplemented with antibiotics (40 mg/L kanamycin and 300 mg/L timentin): Mix $\frac{1}{2}$ MS macronutrients, $\frac{1}{2}$ MS micronutrients, full MS vitamins, 2% (w/v) sucrose, 5 μ M NAA, and 0.1 μ M Kinetin. Adjust pH to 5.7 with KOH and add 0.7% (w/v) Bacto agar. Autoclave to sterilize. Dissolve the antibiotics (powder) in 10 mL water, filter sterilize with 0.2- μ m filter, and add them to the medium.

2.7. Acclimatization

1. Soilless potting medium Metro-Mix 510 (SUN GRO Horticulture, Bellevue, WA, USA), which is a formulation of bark, Canadian Sphagnum peat moss, vermiculite, bark ash, starter nutrient charge (with Gypsum) and slow-release nitrogen, dolomitic limestone, and a wetting agent.
2. Anti-Stress 2000 (Polymer Ag, Fresno, CA). Prepare an aqueous solution (1:20, v/v).

3. Methods

3.1. Seed Collection

1. Select fully ripe plum fruits (see Note 5).
2. Collect the stones by removing as much flesh as possible.
3. Wash the stones in a 20% commercial bleach solution for 30–60 min.
4. Discard the bleach solution, dry the stones, and store at 4°C in a breathable cloth mesh bag(s).

3.2. Explant Preparation

1. Crack the stones and disinfest seeds in a beaker containing a solution of 20% commercial bleach (commercial bleach = 6.15% sodium hypochlorite) and 0.02% Tween-20. Stir the soaking seeds for 20 min using a magnetic stir plate.
2. Rinse three times with sterile ddH₂O in a laminar flow bench (see Note 6).
3. Soak overnight disinfested seeds in sterile ddH₂O at room temperature.
4. Remove seed coats with the aid of a sterile scalpel.
5. Discard the radical and epicotyl of the hypocotyls excised from the zygotic embryos (Fig. 1a).
6. Slice the hypocotyls into three sections (0.5–1 mm) (Fig. 1a) (see Note 7).

3.3. *Agrobacterium* Culture and Preparation

1. Culture the bacterium overnight in 25 mL LB with the proper antibiotics. In this example, 20 mg/L gentamicin for GV3101 chromosomal resistance selection and 50 mg/L kanamycin for pBINPLUS/ARS plasmid selection.
2. Spin down the bacterium ($956 \times g$, 15 min at room temperature).
3. Resuspend the bacteria in 25-mL Bacterial Resuspension Medium (BRM) containing AS to a final concentration of 100 μ M.
4. Incubate the bacteria for 4–5 h in BRM plus AS medium on a 100 rpm shaker at room temperature.
5. For infection, the bacterial suspension may be used without diluting, or it may be diluted to $OD_{600} = 0.3$ – 0.6 .
6. Place the hypocotyl slices in the bacterium suspension for 20 min.
7. Briefly dry the explants and place them on the cocultivation medium (Fig. 1b).

3.4. Co-cultivation

1. After placing the explants on SRM supplemented with 9 μ M 2,4-D, seal the Petri dishes and culture them in a tissue culture room with a 16/8 h light/dark cycle, light intensity of 20–25 μ mol/m²/s (low light intensity), and a temperature of $24 \pm 1^\circ\text{C}$ for 3 days.

3.5. Washing

1. After cocultivation, rinse the explants briefly in liquid washing medium.
2. Dry the explants well on sterile filter paper.
3. Place explants in SRM in Petri plates supplemented with 80 mg/L kanamycin and 300 mg/L timentin (see Note 8).

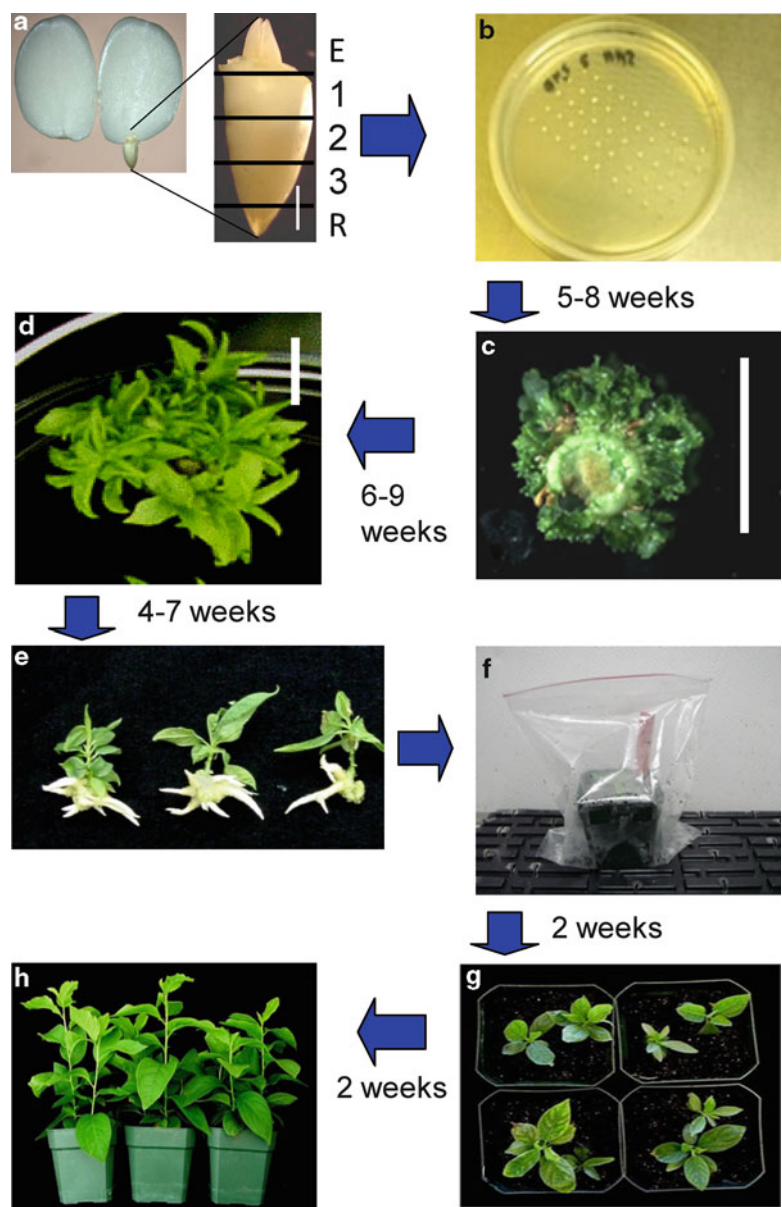


Fig. 1. Regeneration of transgenic plums. (a) Plum hypocotyl slices where 1, 2, and 3 represent the explants used in this study. Epicotyl (E) and radicle (R) were not used. Vertical bar indicates 1 mm. (b) Explants cocultivated for 3 days with *Agrobacterium tumefaciens* in regeneration medium (SRM) supplemented 9 μ M 2,4-D. (c) Adventitious shoot regeneration from hypocotyl slices in antibiotic selection medium. Vertical bar indicates 1 cm. (d) Putative transgenic shoot cluster after cultivation in shoot-growing medium (SGM) supplemented with timentin and kanamycin. Vertical bar indicates 1 cm. (e) Shoots rooted in a rooting medium. (f) Plants introduced in plastic bags provided with zipper seal cultured in a growth chamber. (g) Acclimated rooted plantlets maintained in the growth chamber after the plastic bag is fully opened. (h) Transgenic plants grown in a greenhouse.

3.6. Transgenic Shoot Regeneration

1. Culture explants in a tissue culture room with a 16/8 h light/dark cycle, light intensity of 20–25 $\mu\text{mol}/\text{m}^2/\text{s}$ (low light intensity), and a temperature of $24 \pm 1^\circ\text{C}$ (similar conditions to the cocultivation period).
2. After 4–5 weeks, shoot buds start appearing. Usually, buds appear in clusters.
3. Let the shoot bud clusters develop for 1–2 more weeks (Fig. 1c) and then transfer regenerating explants to SGM in culture jars supplemented with 80 mg/L kanamycin and 300 mg/L timentin for transgenic shoot elongation.
4. Subculture explants that did not show any regeneration to fresh SRM with 80 mg/L kanamycin and 300 mg/L timentin. Eventually, they will regenerate in the subsequent subculture. In this case, repeat step 3.

3.7. Transgenic Shoot Elongation

1. After transferring explant onto SGM, place the plates under a higher light intensity (45–50 $\mu\text{mol}/\text{m}^2/\text{s}$).
2. After 3–4 weeks on SGM, split the explants to separate the shoot clusters (see Note 9).
3. Place the individual clusters in culture jars with SGM supplemented with 80 mg/L kanamycin and 300 mg/L timentin and subculture them every 3 weeks on fresh SGM (Fig. 1d).

3.8. Rooting

1. When shoots elongated from clusters reach 2–3 cm length, separate them from the cluster.
2. Place shoots in RM supplemented with 40 mg/L kanamycin and 300 mg/L timentin.
3. In 3–4 weeks, roots appear. Let the shoots stay in the RM for 1–2 more weeks, for root system development, and then proceed to acclimatization (Fig. 1e).

3.9. Acclimatization

1. Wash rooted shoots in sterile water to eliminate agar residues.
2. Transfer to 3 in-square peat pots containing a soilless potting mixture such as Metro-Mix 510 or a similar product.
3. Introduce potted plantlets into plastic zipper bags and seal (Fig. 1f). Maintain them in a tissue culture growth chamber with a 16/8 h light/dark cycle, light intensity of 45–50 $\mu\text{mol}/\text{m}^2/\text{s}$, and a temperature of $24 \pm 1^\circ\text{C}$ (see Note 10).
4. After 2 weeks, open the plastic bags (Fig. 1g) (see Note 11).
5. Let the potted plants stay 2 more weeks in the growth chamber with the bag fully opened.
6. Transfer plants to a greenhouse and spray them with an aqueous solution (1:20, v/v) of Anti-Stress 2000 (Fig. 1h).

4. Notes

1. Seed extracted from plum stones stored cold (4°C) for up to 3 years can be successfully transformed using this protocol. While “Bluebyrd” seed consistently produce high numbers of transgenic lines, seed of other varieties including “Stanley,” “President,” “Tulare Giant,” and “Sutter” have also produced transgenic lines. Tian et al. (14) report on regeneration, but not transformation, from seeds of a number of plum cultivars that may be candidates for transformation.
2. Autoclave all media at 121°C for 20 min.
3. Timentin is a mixture of two antibiotics: ticarcillin disodium and clavulanic acid in the ratio of 30:1. In case the ratio of these antibiotics is 15:1, add 600 mg/L timentin to SRM to prevent bacterium overgrowth.
4. Stable at 4°C up to 3 months, but we recommend making fresh stock solution after 4 weeks for all growth regulators.
5. Collect the fruit when the seed is completely mature, but the flesh is still firm, so the fruit is not over ripened. This will reduce possible microbial contamination of seeds.
6. From this point until plantlet acclimatization, all steps will be performed in a laminar flow bench under sterile conditions.
7. Try to keep the embryo attached to the cotyledons. This will help in excising the hypocotyl slices. The use of a stereomicroscope is recommended for this step.
8. Culture a maximum of 25 hypocotyl slices per Petri dish of medium in order to allow the enlargement of explants.
9. Collect only the green and vigorous shoot clusters and discard chlorotic or nonvigorous clusters; they probably are escapes or chimeras.
10. During this acclimatization step, water the plants with ddH₂O as needed.
11. The development of new leaves indicates successful acclimatization.

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Chapter 17

Co-transformation of Grapevine Somatic Embryos to Produce Transgenic Plants Free of Marker Genes

Manjul Dutt, Zhijian T. Li, Sadanand A. Dhekney, and Dennis J. Gray

Abstract

A cotransformation system using somatic embryos was developed to produce grapevines free of selectable marker genes. This was achieved by transforming *Vitis vinifera* L. “Thompson Seedless” somatic embryos with a mixture of two *Agrobacterium* strains. The first strain contained a binary plasmid with an *egfp* gene of interest between the T-DNA borders. The second strain harbored the neomycin phosphotransferase (*nptII*) gene for positive selection and the cytosine deaminase (*codA*) gene for negative selection, linked together by a bidirectional dual promoter complex. Our technique included a short positive selection phase of cotransformed somatic embryos on liquid medium containing 100 mg/L kanamycin before subjecting cultures to prolonged negative selection on medium containing 250 mg/L 5-fluorocytosine.

Key words: Grapevine, *Vitis vinifera*, Cotransformation, *codA*, *nptII*, Negative selection, Vegetatively propagated crops

1. Introduction

In transformation studies, selectable marker genes are introduced into the plant genome, usually with a gene of interest, to provide transformed cells a growth advantage to outgrow nontransformed cells in a selection medium (1). In absence of a selectable marker gene, lineages of transformed cells would die out as a consequence of competition from nontransformed cells (2). Thus, selection of transformed cells for their ability to proliferate in the presence of the selective agent allows isolation of transgenic cells containing only the gene of interest (3). However, once a desired transgenic plant is produced from transgenic cells, the marker gene becomes useless (4).

Most marker genes in use today function by conferring transformed cells resistance to selection agents. Among the commonly used marker genes, both *bar* and *pat* genes from *Streptomyces*

hygroscopicus and *S. viridochromogenes*, respectively, encode phosphinothricin acetyltransferase (PAT) and confer resistance to the herbicide phosphinothricin (PPT). Also, the *nptII* gene from *Escherichia coli* encodes neomycin phosphotransferase II and confers resistance to aminoglycoside antibiotics that contain a 3'-hydroxyl group like kanamycin, neomycin, and G418, while the *hptII* gene from *E. coli* encodes hygromycin phosphotransferase and is capable of conferring resistance to the hygromycin B antibiotic.

Several strategies have been proposed to generate marker-free transgenic plants, including use of the following: cotransformation, transposable elements, site-specific recombination, or intrachromosomal recombination (5–10). In a cotransformation system, transformation involves the use of two separate plasmid vectors. One plasmid vector contains only the gene of interest and the other contains both positive and negative selection marker genes within the same T-DNA region (4). After cotransformation, some target cells would only receive gene(s) from one plasmid and some cells would receive genes from both plasmids. The frequency of recovering a desired transformant that contains T-DNA from one or both plasmids can be summed as a function of efficiency of transformation coupled with degree of linkage between insertion sites (11). The cotransformation process is illustrated in Fig. 1.

The premise behind cotransformation-based marker-free system is that transient marker gene expression can cross protect adjacent cells that express only the gene of interest from the lethal effect of selection environment (12). In this system, selectable marker genes and the gene of interest are not physically linked. Instead, they are placed into separate T-DNAs of different plasmids (4, 13) for subsequent independent segregation in a Mendelian fashion (14, 15). A positive selection gene could be linked to a negative selection gene within the same T-DNA region. Following cotransformation, a short-term selection phase based on the positive selection marker would presumably arrest the proliferation of, or even kill, nontransformed cells while allowing the transformed cells to survive. During *Agrobacterium*-mediated transformation, the number of cells to which T-DNA is transferred greatly exceeds, by several orders of magnitude, the number of cells that ultimately become stably transformed. Cotransfer of two different T-DNA molecules from *Agrobacterium* into a single plant cell nucleus would not necessarily lead to cointegration of both (16). Nevertheless, transferred genes could be transiently expressed from nonintegrated T-DNA molecules (17, 18). When positive selection pressure is subsequently removed, some cells would have had opportunity to incorporate the gene of interest but not the selection marker gene into the genome, resulting in marker-free DNA integration. Thus, plant cells could survive a temporary selection phase by virtue of transient expression of a marker gene present in

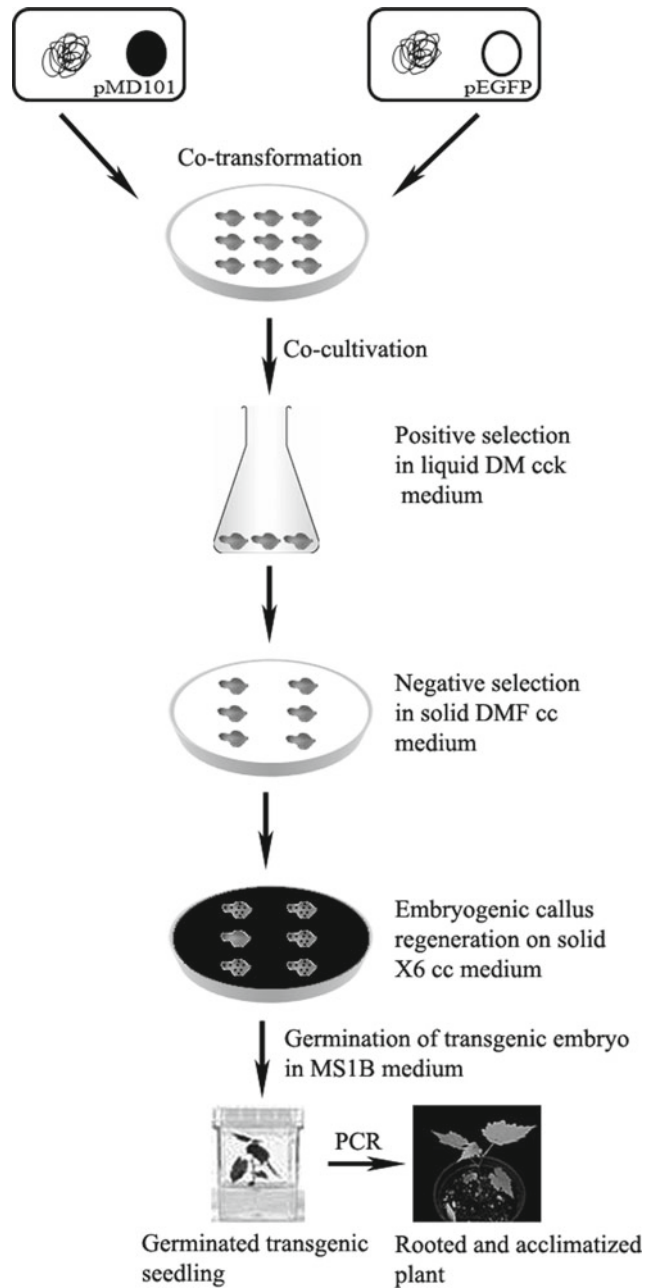


Fig. 1. Schematic representation of the sequence of events in generating marker-free transgenic grapevines. Dotted embryos in the $\times 6$ plate represent transgenic lines.

conventional T-DNA. When a negative selection pressure is applied, cells containing the positive as well as negative selection gene would be killed, leaving cells containing the gene of interest. These cells can then be reprogrammed into plant development under in vitro conditions.

In the negative selection system, expression of a negative selection gene causes either immediate or conditional cell death under specific developmental or environmental conditions and therefore allows for selection of cells lacking these marker genes (19). Thus, combining cotransformation system with negative selection can lead to a practical method for identifying transgenic plants without large-scale PCR (4). A common negative selection system utilizes an *E. coli* gene encoding cytosine deaminase or *codA* (4). Cells expressing cytosine deaminase activity are sensitive to cytosine analogue 5-fluorocytosine (5-FC) since the enzyme deaminates 5-FC to a highly cytotoxic compound 5-fluorouracil (5-FU) (20). Cells lacking cytosine deaminase activity are resistant to 5-FC (21). The ability of *codA* gene to confer cytosine deaminase activity and susceptibility to treatment with 5-FC to transformed cells makes the gene useful as a negative selection marker in transformation studies.

This chapter details the use of a cotransformation-based system for marker-gene-free transformation in grapevines. To achieve this objective, somatic embryos (SE) of *Vitis vinifera* “Thompson Seedless” were cotransformed with two plasmids, one containing an *egfp* gene, which was considered as the gene of interest in this example, and the other containing the negative selection gene (*codA*) and the positive selection gene (*nptII*) linked together by a bidirectional dual promoter complex (22). Marker-free transgenic SEs were identified based on EGFP expression and sensitivity to both positive and negative selection conditions. Polymerase chain reaction (PCR) was carried out to confirm cotransformed clones.

2. Materials

2.1. Tissue Culture Media

1. Shoot initiation medium (C2D4B): C2D (23) salts and vitamins, 30 g/L sucrose, 4 μ M BAP, and 7 g/L TC Agar (see Note 1), pH 5.7.
2. Embryo initiation medium (NB2): Nitsch and Nitsch (24) salts and vitamins, 20 g/L sucrose, 1 μ M BAP, 5 μ M 2,4-D, and 7 g/L TC Agar, pH 5.7.
3. Embryo maintenance medium ($\times 6$): MS medium (25) modified with 3.033 g/L KNO_3 and 0.364 g/L NH_4Cl , 60.0 g/L sucrose, 1.0 g/L *myo*-inositol, 7.0 g/L TC Agar, and 0.5 g/L activated charcoal, pH 5.7. The medium had glycine omitted from the vitamins.
4. Positive selection medium (DM): DKW basal medium (26) supplemented with 0.3 g/L KNO_3 , 1.0 g/L *myo*-inositol, 2.0 mg/L thiamine-HCl, 2.0 mg/L glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 μ M BAP, 2.5 μ M NOA, and

2.5 μM 2,4-D, pH 5.7. 200 mg of carbenicillin and cefotaxime and 100 mg of kanamycin sulfate were added to liquid media.

5. Negative selection medium (DMF): DM medium supplemented with 7.0 g/L TC Agar. 200 mg of carbenicillin and cefotaxime and 250 mg of 5-fluorocytosine (5-FC) were added to precooled media before pouring into 100-mm $\times$ 15-mm Petri dishes (see Note 2).
6. Embryo germination medium (MS1B): MS medium (25) supplemented with 1 μM BAP and 7.0 g/L TC Agar, pH 5.7.

2.2. In Vitro Plant Materials

1. *V. vinifera* L. “Thompson Seedless” plants: 5-cm-long actively growing shoots from plants in a greenhouse (see Note 3).
2. Sterile deionized water.
3. Tween-20.
4. Five percent solution of Clorox® bleach in sterile deionized water.
5. Sterile container.

2.3. Agrobacterium Culture and Growth Media

1. Binary vectors: pMD101 (27) containing a divergent arrangement of *codA* and *nptII* expression cassettes and pEGFP containing an *egfp* gene (see Note 4; Fig. 2).
2. Incubators for bacterial culture.
3. *Agrobacterium tumefaciens* EHA105 stock containing appropriate plasmid (stored in 20% glycerol at -80°C).
4. Solid bacterial growth medium: yeast extract peptone (YEP) medium (10 g/L peptone, 10 g/L yeast extract, and 5 g/L NaCl, pH 7.0) supplemented with 15 g/L TC Agar, 20 mg/L rifampicin, and 100 m/L kanamycin.
5. Liquid bacterial growth medium: YEP medium supplemented with 20 mg/L rifampicin and 100 mg/L kanamycin.

2.4. Antibiotic Stock Solutions

1. Rifampicin: 20 mg of antibiotic dissolved in 1 mL of DMSO (see Note 5).
2. Kanamycin sulfate: 100 mg of antibiotic dissolved in 1 mL of water. The solution sterilized by filtration using a 0.2- μm membrane.
3. Carbenicillin and cefotaxime: 200 mg of each antibiotic dissolved in 1 mL of water. The solution sterilized by filtration using a 0.2- μm membrane.

2.5. PCR Reagents

1. *Taq* DNA polymerase.
2. 10 $\times$ PCR buffer with Mg^{++} .
3. dNTP mixture.

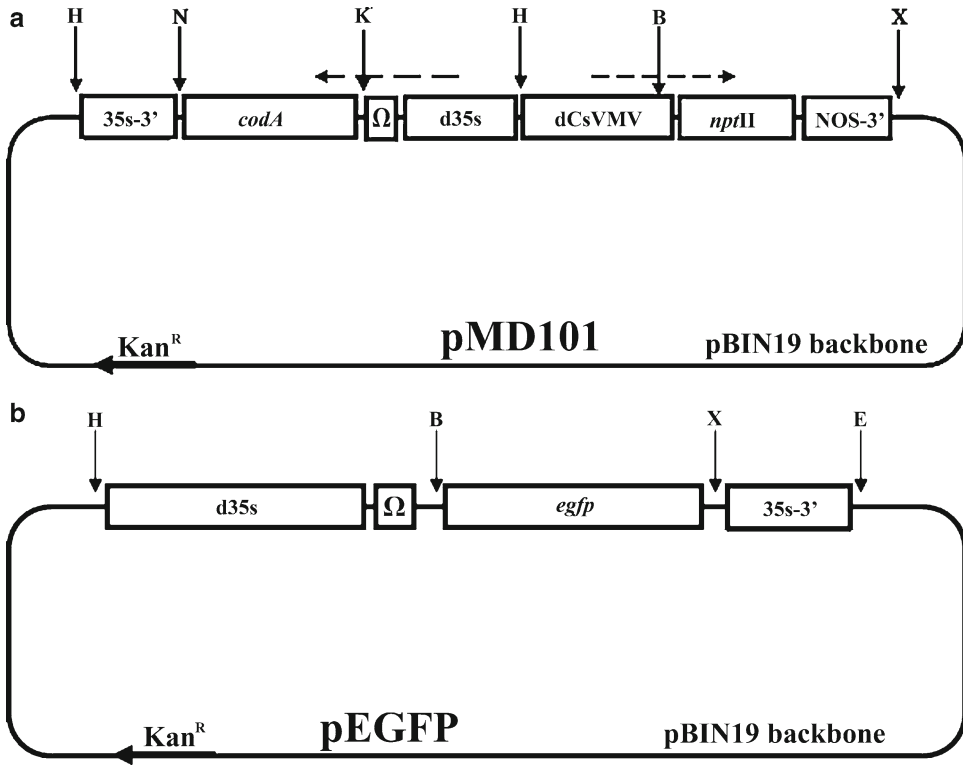


Fig. 2. Schematic representation of the positive/negative selection marker genes containing binary plasmid (pMD101) and the *egfp* containing binary plasmid (pEGFP). Restriction enzyme sites in the plasmid DNA are labeled: *B* *Bam*HI, *E* *Eco*R1, *H* *Hind*III, *K* *Kpn*I, *N* *Not*I, *X* *Xba*I.

4. Primers:

- (a) *egfp*-specific oligonucleotide primer pairs (EG-51), 5'ATG GTG AGC AAG GGC GAG GAG CTG T3' and (EG-32) 5'CTT GTA CAG CTC GTC CAT GCC GAG A3'.
- (b) *codA*-specific oligonucleotide primer pairs (CDA-51), 5'ATG GTA CCA TGT CGA ATA ACG CTT TAC AAA3' and (CDA-32) 5'ATG CGG CCG CCG TCA ACG TTT GTA ATC AAT GGC TTC3'.

2.6. Other Equipment

1. Leica MZFLIII stereomicroscope equipped for epifluorescence with an HBO 100-W mercury lamp illuminator and a GFP filter set composed of an excitation filter (470/40 nm), a dichromatic beam splitter (485 nm), and a barrier filter (525/50 nm) (Leica Microscopy System Ltd., Heerbrugg, Switzerland).
2. Sunrise Optical Microplate Reader (Phenix Research Products, CA, USA).
3. 96-well PTC 100 Thermal Cycler (MJ Research, Watertown, MA).

3. Methods

3.1. Initiation of In Vitro Micropropagation Cultures

1. Harvest actively growing shoots, 5 cm long, from “Thompson Seedless” plants grown in a greenhouse.
2. Wash shoots with deionized water for 5 min.
3. Dissect to 1-cm-long tips inside a laminar flow hood.
4. Wash cut shoots with 70% ethanol for 30 s followed by a quick rinse in sterile double distilled water.
5. Surface sterilize shoots with gentle shaking for 5 min in a 5% solution of Clorox® bleach containing a drop of Tween-20 (see Note 6).
6. Dissect meristem from the shoot tip by removing it along with 2–3 subtending leaf primordia.
7. Place isolated meristems on solidified C2D4B medium supplemented with 20 mg/L rifampicin (see Note 7). Wrap plates in aluminum foil or keep in boxes for 1 week in the dark.
8. After 1 week, transfer cultures for 1 month into a 16-h light/8-h regime at 25°C using cool-white fluorescent lights. Discard any contaminated plates.
9. After a month in culture, excise shoot tips and transfer into fresh medium without rifampicin.
10. Maintain stock plants by subculturing shoot tips onto fresh C2D4B medium every 8 weeks.

3.2. Initiation of In Vitro Embryo Cultures

1. Extract the youngest leaves of in vitro-grown “Thompson Seedless” plants and place them abaxial side down in NB2 medium in dark for 1 month (see Note 8).
2. Transfer cultures to a 16-h light/8-h dark cycle using cool-white fluorescent lights for initiation of proembryonic masses (PEM).
3. Subculture PEM clumps into fresh NB2 medium for another month.
4. Transfer the proliferating PEMs into a plant growth regulator (PGR)-free $\times 6$ embryo maintenance medium. Place Petri dishes under low light levels (see Note 9).

3.3. Agrobacterium Preparation

1. Obtain *Agrobacterium* cultures kept in a -80°C freezer and thaw.
2. Remove a loopful of bacteria from each thawed culture, and streak it on an individual YEP plate (see Note 10).
3. Incubate plates at 26°C for 2 days.
4. Use a single bacterial colony and inoculate a flask of 25 mL liquid YEP medium containing appropriate antibiotics.

5. Culture for 24 h at 26°C.
6. Centrifuge cells at $3,220\times g$ for 8 min at 25°C.
7. Resuspend cells in 25 mL $\times 6$ medium.
8. Incubate cell suspensions for a further 3 h at 26°C (see Note 11).
9. Prior to use in transformation, measure the optical density (OD) of cultures, and adjust to 0.8 (see Note 12).

3.4. Cotransformation of Grapevine SE

The general outline for the cotransformation-based marker-gene-free transformation in grapevines is illustrated in Fig. 1.

1. Grapevine SEs in midcotyledonary stage of development should be used (see Note 13).
2. Mix 1 mL of each of two *Agrobacterium* strains in a 1:1 ratio (see Note 14).
3. Submerge grapevine SEs in bacterial solution for 10 min.
4. Blot SEs on sterile paper towels and transfer onto filter papers wetted with liquid DM (see Note 15).
5. Cocultivate SEs and *Agrobacterium* by keeping Petri dishes in dark at 26°C for 3 days.

3.5. Primary Selection of Putative Transformed Embryos

1. Transfer SEs into 50 mL liquid DM medium containing 200 mg/L each of carbenicillin and cefotaxime and culture on an orbital shaker at 120 rpm for 5 days at 26°C for initial positive selection (see Note 16).
2. Transfer SEs into DMF medium for subsequent negative selection. Petri dishes containing SEs can be placed in the dark for 1 month.
3. Transfer developing calli into fresh DMF medium and culture for another month.
4. Check for embryogenic calli formation and transfer such calli onto $\times 6$ cc medium for SE proliferation.

3.6. Secondary Selection of Putative Transformed Embryos

1. After 1 month of transfer of embryogenic calli, SE development can be observed.
2. Stable expression of the gene of interest (EGFP in this case) can be determined based on the formation of GFP-expressing calli and SEs proliferating in $\times 6$ medium.
3. Transfer EGFP-positive calli to fresh $\times 6$ medium for further proliferation and induction of transgenic SE.
4. Isolate transgenic SE from a particular primary SE explant and designate as an independent transgenic line (see Note 17).

3.7. Germination of Transgenic Embryos and Growth of Plants

1. Place mature transgenic SE in MS1B medium and maintain at 25°C with a 16-h light/8-h dark cycle using cool-white fluorescent lights.
2. Well-developed transgenic plants with an actively growing root system can be subsequently hardened off in plug trays containing Promix BX potting medium (A.H. Hummert Seed Co., MO, USA).
3. Plants that had produced three or more leaves can be transferred to a greenhouse and maintained in 17-cm × 16-cm pots filled with Promix BX (Premier Horticulture Inc., PA, USA).

3.8. PCR Analysis of Transgenic Plants

1. Duplex PCR (Fig. 3) from leaves of transgenic plants can be carried out to:
 - (a) Confirm genomic integration of the *egfp* gene using *egfp*-specific oligonucleotide primer pairs (EG-51), 5'ATG GTG AGC AAG GGC GAG GAG CTG T3' and (EG-32) 5'CTT GTA CAG CTC GTC CAT GCC GAG A3'.
 - (b) Confirm the absence of the *codA* gene using *codA*-specific oligonucleotide primer pairs (CDA-51), 5'ATG GTA CCA TGT CGA ATA ACG CTT TAC AAA3' and (CDA-32) 5'ATG CGG CCG CCG TCA ACG TTT GTA ATC AAT GGC TTC3'.

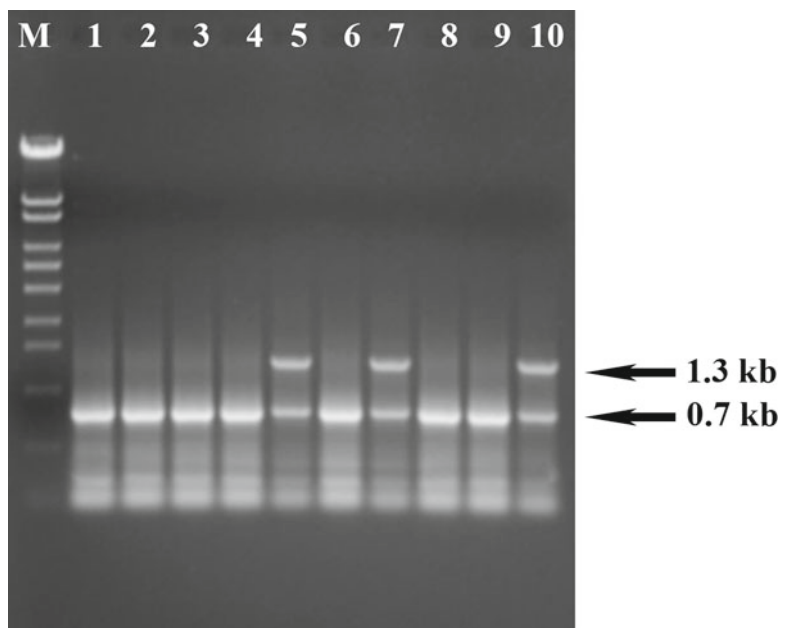


Fig. 3. PCR amplification products of genomic DNA of transgenic marker-free grapevines containing the *egfp* gene. Amplification was carried out using *codA* (1.3-kb product) and *egfp* (0.7-kb product) specific primers. Several transgenic lines that contained only the gene of interest were detected.

2. Isolate total DNA from transgenic plants using the CTAB protocol (28) (see Note 18).
3. To make a 30- μ L PCR reaction, mix add the following components: 2 μ L of 25 ng/ μ L genomic DNA, 6.5 μ L of water, 3 μ L of 10 $\times$ buffer, 4 μ L of 1.25 mM deoxynucleotides, 2 μ L of 25 mM MgCl₂, 3 μ L of 20 pM/mL of each of the primers, and 0.5 μ L of 5 U/ μ L Taq polymerase.
4. Perform PCR with the following reaction parameters: 1 cycle at 95°C for 5 min, 30 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 5 min.
5. Load PCR products on a 0.6% agarose gel containing 5 μ L of 1 mg/mL ethidium bromide in 1 $\times$ TAE buffer. The gel can be subjected to a 60-V current for 40 min, and DNA bands, visualized with a UV transilluminator.

4. Notes

1. Different gelling agents were evaluated for grapevine tissue culture, and it was determined that TC Agar provided the most consistent results. It is recommended to use TC Agar for optimum growth and performance of in vitro cultures.
2. 5-FC is purchased from Sigma-Aldrich. The salt can be completely dissolved in water by warming to 65°C for a few minutes followed by vortexing. 250 mg/L of 5-FC was determined to be the lowest concentration at which complete inhibition of growth could be achieved in SE and was thus used in subsequent experiments.
3. “Thompson Seedless” grapevine plants that are used as mother plants for the initiation of in vitro cultures have to be in a good ontological state, free from diseases and pests. Shoots used should be tender and not lignified.
4. Any binary vector containing a gene of interest without a selectable marker gene present in the T-DNA can be used as the gene of interest to be incorporated into the plant’s genome.
5. All antibiotic stocks (with the exception of rifampicin) are filter sterilized and stored in 1-mL aliquot stocks in Eppendorf tubes at –20°C. Antibiotics stored in this manner can stay active for at least 3 months. It is recommended to thaw antibiotic solutions once. Any leftover solutions are best discarded.
6. It is recommended not to exceed a 5% solution of Clorox® bleach for surface sterilization of greenhouse material. It is also important to add a drop of Tween-20 or any suitable polysorbate surfactant or detergent.

7. Rifampicin is added to initial C2D4B media formulation to inhibit potential endophytic bacteria. Because rifampicin is light labile, a preliminary 72-h growth phase in dark is necessary.
8. Embryogenic potential is best in the youngest, unopened leaves just below the apical meristem. The potential decreases as the age of leaves increase.
9. Transfer to a PGR-free medium is necessary for initiation and maintenance of SEs. Maintenance under low light levels has been observed to be beneficial for proliferation of SEs.
10. *Agrobacterium* cultures can be directly initiated from thawed glycerol stock. However, cultures initiated from colonies streaked on a YEP plate are usually more vigorous and grow faster.
11. Three hours of incubation has been determined to be optimum to allow most of the cells to be in the log phase of growth. Cells in this phase possess good infection ability.
12. OD of cultures is measured in a Sunrise Optical Microplate Reader set to read at 620 nm. The initial OD of cultures is usually in-between 1 and 1.5 and requires dilution with $\times 6$ medium.
13. It is important for optimum results that embryogenic cultures in the midcotyledonary stage of growth are used. The best results are obtained from cultures that have been subcultured into $\times 6$ medium within the last 2 months. Cultures that are advanced in age do not provide good results.
14. In preliminary experiments, several plasmid ratios (pMD101: pEGFP) of 1:0, 0:1, 1:1, 2:1, or 1:2 were evaluated. It was determined that 1:1 was optimum for cotransformation.
15. Filter papers soaked in liquid DM are used for cocultivation as this results in lower *Agrobacterium* overgrowth on treated SEs when compared to cocultivation in solidified DM plates.
16. In preliminary studies, initial positive selection consisted of a temporary positive selection phase in either liquid or solidified DM_{check} medium for 3, 5, or 7 days. It was determined that there was no significant difference between 3, 5, and 7 days in liquid or solidified medium. In general, cotransformed embryos subjected to 3 or 5 days of positive selection in liquid medium treatments had a higher percentage of EGFP expression than those from solid medium treatments.
17. It is necessary to carefully isolate a single transgenic embryo from each clump and transfer to $\times 6$ medium for further growth and development. This prevents the introduction of multiple transgenic events which could have resulted from transformed sister cells. On subculture to fresh $\times 6$ medium, the primary embryo produces embryogenic cell masses and regenerates

numerous secondary somatic embryos, both from the cell masses and from hypocotyl cells.

18. We use the CTAB protocol to isolate DNA. However, any commercial kit can be used for DNA isolation.

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Initiation and Transformation of Grapevine Embryogenic Cultures

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Abstract

Protocols for the production and transformation of grapevine embryogenic cultures are described. Embryogenic cultures are initiated from leaves or stamens and pistils and transformed with *Agrobacterium* containing an enhanced green fluorescent protein/neomycin phosphotransferase II (*egfp/nptII*) fusion gene. Cultures are transferred to induction medium in the dark for callus formation and proliferation. Resulting cultures are transferred to somatic embryo development medium to induce secondary embryogenesis and formation of transgenic somatic embryos. Transgenic embryos identified on the basis on GFP fluorescence and kanamycin resistance are transferred to germination medium to regenerate transgenic plants. The presence of transgenes in independent plant lines is confirmed by PCR.

Key words: *Vitis*, Somatic embryogenesis, *Agrobacterium*, Transgenic plants, Transgenes, Culture medium, Growth regulators, Plant tissue culture

1. Introduction

Grape is a high-value crop, prized for its multiple uses as a fresh fruit and processed food product (jelly, juice, raisins, and wine). The health attributes of grape have become increasingly well documented. A host of antioxidants and vitamins found in grape and its products help prevent heart disease and cancer. These attributes have contributed to make grape the world's most important fruit crop, including a major crop in the United States (1) where it contributes \$162 billion annually to the US economy (2).

Vitis crop improvement is accomplished by clonal selection of spontaneous bud mutations and breeding (3, 4). However, random occurrence of bud mutations limits directed crop improvement. Breeding has limited application for genetic improvement due to extreme heterozygosity of the *Vitis* genome, which is fostered by inbreeding depression (5). Inbreeding depression makes

backcrossing and recurrent selection difficult. In particular, for varieties used in wine production where the enological characteristics are finely appreciated, it is impossible to introduce a useful trait via breeding without disrupting the desired phenotype. In addition, the long juvenile period of vines makes screening of new selections tedious and time consuming (6).

Genetic transformation involves transfer of a DNA sequence into a plant cell and its subsequent integration into the host genome. Transformation serves as an important tool in plant biology for studying gene function and expression. It also offers a potential alternative for adding single traits, such as disease resistance, to elite grapevine varieties without changing desirable characteristics (7). The essential prerequisites for a gene transfer system include the availability of a target tissue, a method to introduce foreign DNA, and a procedure to select transformed cells and regenerate transgenic plants (8).

Grapevine embryogenic cultures have been the most-used target tissue for transgene insertion and recovery of transgenic plant lines (9–14). The routine use of embryogenic cultures for transformation (7) necessitates optimization of protocols for culture initiation and maintenance. We have optimized protocols for initiation and maintenance of embryogenic cultures for several *Vitis* species and varieties (15, 16). Cultures can be initiated from leaves or floral explants and can be maintained on hormone-free embryo development medium for extended periods of time. Somatic embryos at the cotyledonary stage of development are used as target tissues for genetic transformation and plant regeneration.

Although biolistic bombardment has been used for delivery of transgenes into grape plant cells (17), *Agrobacterium*-mediated transformation is by far the most commonly used method for producing transgenic plants (18). In nature, *Agrobacterium tumefaciens* is a pathogenic bacterium infecting grapevine and transfers a part of its DNA (T-DNA), present on a tumor-inducing plasmid (Ti plasmid), to the nuclear genome of the host plant. Genes coding for the synthesis of amino acids known as opines are present on the T-DNA. The production of opines results in the proliferation of plant cells and formation of tumors (19). This T-DNA transfer mechanism is exploited for transformation of grapevines by replacing tumor-causing genes on the T-DNA with desired genes of interest to be studied. Such strains can no longer cause tumors upon infection and are termed “disarmed” strains. Genes of interest either can be inserted into the T-DNA region of a Ti plasmid or the T-DNA region of a smaller plasmid that is then introduced into disarmed *Agrobacterium*. The latter, known as a binary vector system, is most commonly adopted for transformation (20).

The success of a transformation system depends on the ability to successfully recover transformants after cocultivation. Certain genes are incorporated, along with the gene of interest, into the

T-DNA region to confirm the presence of the T-DNA in plant cells and/or confer a competitive advantage to growth of transgenic cells over nontransformed cells. These include reporter genes, which indicate the presence of the T-DNA, and selectable marker genes that most often confer antibiotic resistance to transgenic cells. The green fluorescent protein (GFP) gene isolated from the Pacific jellyfish *Aequoria victoria* is routinely used as a reporter gene in grapevine transformation studies (10, 11). Plant cells expressing GFP produce a bright green fluorescence, which can be observed under a stereomicroscope equipped for epifluorescence illumination. Alternatively, GFP can be visualized in plant cells using a relatively inexpensive detection system (21). Selectable marker genes such as neomycin phosphotransferase II (*npt II*) and hygromycin phosphotransferase (*hpt*) genes are frequently used along with reporter genes in genetic transformation (22, 23). Transgenic cells carrying these marker genes can selectively grow on the culture medium containing kanamycin or hygromycin antibiotics, while inhibiting the growth of nontransformed cells.

This chapter presents methods for the production and transformation of embryogenic cultures to regenerate transgenic plants with desired genes of interest. Embryogenic cultures initiated either from leaves or floral explants are cocultivated with *Agrobacterium* containing a binary plasmid with the genes of interest. Transformed somatic embryo lines are identified based on GFP fluorescence and kanamycin resistance. Regenerated plants obtained from germinated transgenic embryos are hardened in a growth room and transferred to a greenhouse. Presence of transgenes is estimated using polymerase chain reaction (PCR).

2. Materials

2.1. Supplies and Equipment

1. Laminar flow hood.
2. 100-mm × 15-mm plastic Petri dishes.
3. 50-mL centrifuge tube.
4. 125-mL conical flasks.
5. GA-7 Magenta vessel.
6. Scalpels.
7. Forceps.
8. Sterile Whatman 3MM filter paper.
9. Orbital shaker.
10. Centrifuge.
11. Clorox®.
12. Bead sterilizer.

13. Micropipettors and micropipette tips.
14. 70% ethanol.
15. Tween 20.
16. 96-well PTC-100 programmable thermal controller (MJ Research, Watertown, MA).
17. 500- μ L centrifuge tubes.
18. Growth chamber.
19. Leica MZFLIII stereomicroscope equipped for epifluorescence with an HBO 100-W mercury lamp illuminator and a GFP filter set composed of an excitation filter (470/40 nm), a dichromatic beam splitter (485 nm), and a barrier filter (525/50 nm) (Leica Microscopy System Ltd., Heerbrugg, Switzerland).
20. Sunrise optical microplate reader (Phenix Research Products, CA, USA).

2.2. Plant Tissue Culture Media

1. Embryogenic culture induction medium from leaves (NB2 medium), pH 6.0: (28) salts and vitamins, 20 g/L sucrose, 0.1 g/L myo-inositol 1.0 μ M BAP, 5.0 μ M 2,4-D, and 7.0 g/L TC agar (see Note 1).
2. Embryogenic culture induction medium from floral explants (MSI medium), pH 6.0: (27) salts and vitamins, 20 g/L sucrose, 0.1 g/L myo-inositol, 4.5 μ M BAP, 5.0 μ M 2,4-D, and 7 g/L TC agar (see Note 2).
3. Embryogenic culture induction medium from floral explants (PIV medium), pH 5.7: (28) salts, B5 vitamins, 60 g/L sucrose, 8.9 μ M BAP, 4.5 μ M 2,4-D, and 3.0 g/L Phytagel (see Note 3).
4. Somatic embryo (SE) development and maintenance medium ($\times$ 6 medium), pH 5.8: MS medium lacking glycine and supplemented with 3.033 g/L KNO_3 and 0.364 g/L NH_4Cl (as the sole nitrogen source), 60.0 g/L sucrose, 1.0 g/L myo-inositol, 7.0 g/L TC agar, and 0.5 g/L activated charcoal (see Note 4).
5. Liquid cocultivation medium (DM medium), pH 5.7: DKW basal salts (26), 0.3 g/L KNO_3 , 1.0 g/L myo-inositol, 2.0 mg/L each of thiamine-HCl and glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 μ M BA, and 2.5 μ M each NOA and 2,4-D.
6. Callus induction medium (DM medium), pH 5.7: DKW basal salts (26), 0.3 g/L KNO_3 , 1.0 g/L myo-inositol, 2.0 mg/L each of thiamine-HCl and glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 μ M BA, 2.5 μ M each NOA, 2,4-D,

7.0 g/L TC agar, 200 mg/L each of carbenicillin and cefotaxime, and 100 mg/L kanamycin.

7. Somatic embryo germination medium (MS1B), pH 5.8: MS salts and vitamins, 20.0 g/L sucrose, 0.1 g/L myo-inositol, 1.0 μ M BAP, and 7.0 g/L TC agar.
8. Solid *Agrobacterium* culture medium: yeast extract peptone (YEP) medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 15 g/L Bacto agar.
9. Liquid MG/L medium, pH 7.0: 5.0 g/L mannitol, 1.0 g/L L-glutamate, – 5.0 g/L tryptone, 2.5 g/L yeast extract, 5.0 g/L NaCl, 150.0 mg/L KH_2PO_4 , 100.0 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.5 mL/L Fe-EDTA (To make a stock solution of Fe-EDTA, dissolve 7.44 g of $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 1.86 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in sterile distilled water and make final volume to 1 L.) (see Note 5).
10. Liquid 2 $\times$ medium, pH 5.8: $\times 6$ medium modified to contain 20.0 g/L sucrose without TC agar and activated charcoal.

2.3. In Vitro Plant Materials

1. In vitro micropropagation cultures (see Note 6).
2. Dormant vine cuttings (see Note 7).

2.4. Antibiotic Stock Solutions

1. Rifampicin: 20 mg of antibiotic dissolved in 500 μ L of methanol (see Note 8).
2. Kanamycin sulfate: 100 mg of antibiotic dissolved in 1 mL of water and filter-sterilized using a 0.2- μ m membrane.
3. Carbenicillin and cefotaxime: 200 mg of each antibiotic dissolved in 1 mL of water and filter-sterilized using a 0.2- μ m membrane.

2.5. Agrobacterium Culture

1. Binary vector containing an *egfp/nptII* fusion gene under the control of a double cassava vein mosaic virus (CsVMV) promoter.
2. *Agrobacterium* culture stock containing the binary vector (stored in glycerol at -70°C).

2.6. PCR Reagents

1. *Taq* DNA polymerase.
2. 10 $\times$ PCR buffer with Mg^{2+} .
3. dNTP mixture.
4. Primers
 $egfp$ -specific oligonucleotide primer pairs EG-51 (5'-ATG GTG AGC AAG GGC GAG GAG CTG T-3') and EG-32 (5'-CTT GTA CAG CTC GTC CAT GCC GAG A-3').

3. Methods

3.1. Initiation of Embryogenic Cultures from Leaves

1. Excise 1.5–5.0-mm-long unopened leaves from in vitro micro-propagation cultures and place five leaves on a Petri dish containing NB2 medium (see Note 9).
2. Cover Petri dishes in aluminum foil and incubate in darkness at 26°C for 5–7 weeks.
3. Transfer Petri dishes containing the callus cultures to cool white fluorescent light ($65 \mu\text{m}^2/\text{s}$ and 16-h photoperiod) at 26°C for 5 weeks. Screen callus cultures at weekly intervals.
4. Explants will produce sectors of compact, cream-colored embryogenic callus and loose, brown-colored nonembryogenic callus (see Note 10).
5. Transfer embryogenic callus to growth regulator free $\times 6$ medium for the development and proliferation of proembryonic masses (PEM) and somatic embryos (SE).
6. Maintain embryogenic cultures by transferring gray-colored PEM to fresh $\times 6$ medium at 4–6 week intervals.
7. Use cotyledonary stage SE for *Agrobacterium*-mediated transformation.

3.2. Initiation of Embryogenic Cultures from Floral Explants

1. Obtain inflorescences from either field grown grapevines or dormant vine cuttings (see Note 11).
2. Determine developmental stages of stamens and pistils and select the optimum stage that produces an embryogenic response (see Note 12).
3. Immerse inflorescences in 70% ethanol for 30 s followed by washing them in distilled water for 30 s.
4. Wash inflorescences in 25% NaOCl solution containing one drop Triton X-100 with constant agitation for 5 min, followed by three, 5-min washes in sterile distilled water.
5. Carefully separate stamens (anthers with intact filaments) from the calyptra and the pistil before placing them on MSI or PIV medium. Also, place the pistil with the remaining filament stubs on the medium. Place 35 stamens in a clump in the center of a Petri dish and five pistils near the perimeter.
6. Seal Petri dishes with Parafilm® and place in darkness at 26°C for 4 weeks.
7. After 4 weeks, transfer Petri dishes under cool white fluorescent lights ($65 \mu\text{mol}/\text{m}^2/\text{s}$ and 16-h photoperiod). Screen developing cultures using a microscope for the presence of embryogenic callus at weekly intervals for 16 weeks.

8. Production of embryogenic callus will be observed from the filament tip or connective tissue of anthers and in some cases from pistils (15).
9. Transfer embryogenic callus to $\times 6$ medium for development and proliferation of SE. Maintain embryogenic cultures by transferring gray-colored PEM to fresh $\times 6$ medium at 4–6 week intervals (see Note 13).

3.3. Initiation of *Agrobacterium* Culture

1. Transfer *Agrobacterium* culture containing the binary plasmid from -80°C to room temperature and thaw.
2. Spread approximately 20 μL of bacterial culture on a Petri dish containing solid YEP medium with 20 mg/L rifampicin and 100 mg/L kanamycin. Incubate dishes in the dark at 26°C for 3 days.
3. Isolate a single colony growing on YEP medium and transfer it to a 125-mL conical flask containing 30 mL MG/L medium with 20 mg/L rifampicin and 100 mg/L kanamycin.
4. Incubate on a rotary shaker at 180 rpm at 26°C for 16–20 h. The bacterial culture should appear cloudy at the end of the culture period.
5. Transfer the culture to a 50-mL centrifuge tube and spin at $2,220\times g$ for 8 min at room temperature. Discard the supernatant and resuspend the pellet in 30 mL liquid $2\times$ medium. Adjust OD value to 0.6 using liquid $2\times$ medium.
6. Transfer the contents of the tube to a 125-mL conical flask and incubate for an additional 4 h on a rotary shaker under the same conditions as above. Use this culture for cocultivation.

3.4. Transformation of Somatic Embryos

1. Transfer cotyledonary stage SE to sterile Petri dishes. Avoid wounding SE during transfer to prevent browning (see Note 14).
2. Add 5.0 mL *Agrobacterium* culture to the SE and mix thoroughly by swirling. Incubate for 7 min. Blot SE dry on filter paper to remove the bacteria.
3. Transfer blotted SE to a Petri dish containing two layers of filter paper soaked in liquid DM medium (see Note 15).
4. Seal the Petri dish with Parafilm® and cocultivate in darkness at 26°C for 3 days.
5. After 3 days, observe SE for transient GFP expression using a Leica MZFLIII stereomicroscope equipped for epifluorescence.
6. Transfer cocultivated SE to a 125-mL conical flask containing liquid DM medium with 200 mg/L each of carbenicillin and cefotaxime and 15 mg/L kanamycin.
7. Transfer the flask to a rotary shaker at 110 rpm and wash SE for 3 days to eliminate bacterial cells.

8. Transfer washed SE to each 100-mm × 15-mm Petri dish containing 25 mL solid DM medium with 200 mg/L each of carbenicillin and cefotaxime and 100 mg/L kanamycin.
9. Place Petri dishes in dark at 26°C for 4 weeks to permit callus development and proliferation.
10. After 4 weeks, transfer callus cultures to 100-mm × 15-mm Petri dishes containing 30 mL ×6 medium with 200 mg/L each of carbenicillin and cefotaxime and 70 mg/L kanamycin for secondary embryo development. Place Petri dishes in dark and screen at weekly intervals for the presence of transgenic SE lines.
11. Independent SE lines can be identified by bright GFP fluorescence and can be used to separate transgenic lines from nontransformed SE.
12. Designate each transgenic SE line as an independent event and transfer to fresh ×6 medium with 200 mg/L each of carbenicillin and cefotaxime and 70 mg/L kanamycin.

3.5. Germination of SE Lines and Plant Regeneration

1. Transfer five SE at the late cotyledonary stage from each independent transgenic line to 100-mm × 15-mm Petri dishes containing 25 mL MS1B medium. Place Petri dishes under cool white fluorescent lights at 65 $\mu\text{mol}/\text{m}^2/\text{s}$ and 16-h photoperiod.
2. After 2 weeks of culture, when SE become enlarged and pigmented, excise the enlarged cotyledons to enhance subsequent shoot development (see Note 16).
3. Transfer resulting plants having a robust root and shoot system to Magenta GA-7 vessels containing 30 mL MS medium. Place vessels under cool white fluorescent lights at 65 $\mu\text{mol}/\text{m}^2/\text{s}$ and 16-h photoperiod for further of transgenic plant lines and a nontransformed plant using CTAB development.
4. Transfer plants to 7-cm plastic pots containing Pro-Mix BX potting mix (Premier Horticulture Inc., Red Hill, PA) and acclimate in a growth room for 2 weeks before transfer to a greenhouse (see Note 17).

3.6. PCR Analysis of Transgenic Plants

1. Isolate genomic DNA from leaves using CTAB protocol (24).
2. Make a 10 μL reaction mixture containing 1.0 μL each of the forward and reverse primer, 1.0 μL genomic DNA (concentration 20 ng/ μL), 1.0 μL 10× buffer, 0.6 μL MgCl_2 , 1.6 μL dNTP (concentration 1.25 mM), 0.1 μL Taq polymerase, and 3.7 μL water.
3. Conduct PCR reactions with one cycle at 95°C for 4 min, 39 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 4 min.

4. Use plasmid DNA as a positive control and DNA from nontransformed plants as a negative control.
5. Load PCR products on a 0.6% agarose gel containing 1.0 μ L of ethidium bromide (1.0 mg/mL). Run the PCR products at 100-V current for 30 min in a gel electrophoresis unit and observe separated DNA bands with a UV transilluminator.

4. Notes

1. Recipes for media are per liter final volume. Media are autoclaved at 121°C and 15 psi pressure for 20 min. pH was adjusted using KOH prior to autoclaving.
2. Embryogenic response from leaves and floral explants is genotype dependent. In general, a greater number of varieties produce embryogenic cultures from floral explants than leaves. This factor needs to be considered prior to culture initiation from a specific explant.
3. Production of embryogenic response on MSI and PIV medium is genotype dependent and needs to be standardized for the genotype being studied.
4. An important factor affecting maintenance of embryogenic cultures is the use of TC agar as a gelling agent. Use of other gelling agents including Bacto agar and Phytigel in $\times 6$ medium results in a rapid decline in embryogenic competence and eventual termination of cultures.
5. Although several bacterial media have been used for culture of *Agrobacterium*, MG/L tends to yield better cell growth and a consistent culture quality. Therefore, we recommend this medium for routine use in transformation of grapevine.
6. In vitro micropropagation cultures can be established from shoot tips of rapidly growing field grapevines on C2D4B medium following the protocol established by Gray and Benton (25). Once established, unopened leaf explants can be obtained from these cultures.
7. Dormant vine cuttings can be obtained after pruning of grapevines during the winter season. Alternatively, cuttings can be obtained from the Foundation Plant Services, University of California, Davis, CA.
8. Antibiotic stock solutions are filter-sterilized and stored at -20°C. Solutions are thawed just prior to use. Antibiotics are added to culture medium after autoclaving and cooling the medium to 55°C. Rifampicin is light sensitive and should be stored and used away from light.

9. We use *V. vinifera* “Thompson Seedless” in the majority of our experiments because of its high embryogenic potential from leaves and ability to recover a large number of independent transgenic plant lines (up to 150 lines from 1.0 g of embryogenic culture) after transformation.
10. A positive embryogenic response can be obtained only from unopened leaves, 1.5–5.0 mm in size. Larger size leaves will produce a nonembryogenic callus and should not be used to initiate cultures.
11. Dormant vine cuttings can be forced to flower by placing them in 500-mL Erlenmeyer flasks containing 250 mL sterile distilled water under cool white fluorescent lights ($65 \mu\text{mol}/\text{m}^2/\text{s}$ and 16-h photoperiod), at 26°C for 3 weeks. Replace the water and trim the basal 1 cm of each cutting at weekly intervals to avoid fungal contamination.
12. The developmental stage of stamens and pistils suitable for obtaining an embryogenic response is variety dependent. In general, stage II and stage III stamens and pistils (15) produce the best embryogenic response among a large number of varieties.
13. It is critical to transfer embryogenic cultures to fresh $\times 6$ medium at 4–6 week intervals. Failure to do so will result in a decrease in regeneration potential of SE and subsequent reduction in transformation efficiency and plant regeneration.
14. Grapevine SE proliferates by direct secondary embryogenesis with new embryos emerging from epidermal or subepidermal cells of primary SE. Thus, surface cells of cotyledonary stage SE are the best target tissues for *Agrobacterium*-mediated transformation.
15. Cocultivation of SE on filter paper is beneficial as it reduces over growth of bacterial cells resulting in a dramatic decrease in cell necrosis and higher transformation efficiency.
16. Cotyledon excision to improve shoot recovery from germinated SE is variety dependent and needs to be tested for each variety being studied.
17. Transformation efficiency varies among *Vitis* species and varieties (18). The highest recovery of transgenic plant lines has been recorded in *V. vinifera* “Thompson Seedless.”

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Chapter 19

Development of Highly Efficient Genetic Transformation Protocols for Table Grape Sugraone and Crimson Seedless

Mercedes Dabauza and Leonardo Velasco

Abstract

Genetic engineering of grapevine is a powerful tool to study gene function as well as to introduce new traits into existing *Vitis* cultivars without altering their essential characters and identity. *Agrobacterium*-mediated transformation is one of the most efficient methods for gene transfer, but the efficiency of the procedure depends on several parameters such as the grapevine genotype, the selection strategy, the *Agrobacterium* strain, and concentration used to infect as well as the culture method among others. This chapter describes highly efficient genetic transformation protocols for seedless table grapevine cultivars Sugraone and Crimson Seedless by co-culturing embryogenic calli with *Agrobacterium tumefaciens*. The procedures are specific for each cultivar by adjusting the kanamycin concentration used to select transformed cells (20 mg/L and 50 mg/L kanamycin for Crimson Seedless and Sugraone, respectively) and the low *Agrobacterium* density used to infect the embryogenic calli (0.06 OD₆₀₀ being more effective for the transformation of Crimson Seedless and 0.2 OD₆₀₀ for Sugraone). Other factors that affect the transformation efficiency are the initial amount of embryogenic calli used to co-culture with *Agrobacterium* and the culture method of calli.

Key words: *Vitis vinifera*, Transgenic plants, *Agrobacterium tumefaciens*, Kanamycin, Bacterial concentration

1. Introduction

Genetic engineering of grapevine is a powerful tool to study gene function as well as to introduce disease resistance, product quality, and production efficiency traits without altering the essential characters and identity of the cultivar in question (1). Although grapevine has been genetically transformed with both biolistics and *Agrobacterium* methods using mainly embryogenic calli (2–4), the transformation and regeneration of transgenic grapevine plants from somatic embryos remains a problem (5) at least for some

economically important cultivars in which the development of specific genetic transformation systems would allow molecular breeding purposes and functional genomic studies.

The efficiency of the *Agrobacterium*-mediated transformation protocols depends on many factors including the grapevine genotype used (6–8), the selection strategies (3, 9–13), the *Agrobacterium* strain (7, 14, 15), the culture method (6, 16), and whether or not antioxidants are used (3, 17, 18). The bacterial concentration is also an important factor affecting transformation efficiency (3, 19, 20).

The infection ability of different *Agrobacterium* strains could be related with their different genetic background (15). Baribault et al. (14) studied the effect of some strains (LBA4404, GV3101, EHA101) and obtained stable expression and integration of the *nptII* gene in calli co-cultured with LBA4404 and EHA101 strains. Torregrosa et al. (7) employed four different *Agrobacterium* strains (EHA105, AGL0, AGL1, LBA 4404) and found that EHA105 showed higher transformation efficiency than LBA4404, the most widely used strain. Wang et al. (12) achieved the regeneration of transgenic plants (25 ± 5) of *Vitis vinifera* “Red Globe” using EHA105. On the other hand, a low plant regeneration rate could be due to a hypersensitivity-like reaction of plant tissue to *Agrobacterium* (3, 21).

This chapter presents a cultivar-specific method to genetically transform two table grapevine cultivars, Crimson Seedless and Sugraone, by co-culturing embryogenic calli with *Agrobacterium tumefaciens* and by adjusting the most suitable kanamycin concentration to select transformed cells as well as the *Agrobacterium* density used to calli infection.

2. Materials

2.1. Plant Material

Embryogenic calli of *V. vinifera* L. cv. Sugraone (Superior Seedless®) and cv. Crimson Seedless, induced from immature anthers and maintained at 25°C in dark by monthly transfers onto fresh 4/1.3 culture medium (22).

2.2. Tissue Culture

2.2.1. Supplies and Equipment

1. Forceps and scalpels.
2. Sterile, disposable Petri dishes.
3. Sterile filter paper.
4. Autoclaved nylon meshes (0.1 mm² pore size) of 85-mm diameter.
5. Sterile pipette tips: 5 mL, 1 mL, and 200 µL.
6. Autoclaved Erlenmeyer flasks of 250 mL and 100 mL capped with cotton and aluminium foil.

7. Bunsen burners.
8. Inoculation loops.
9. Sterile 50-mL conical polypropylene centrifuge tubes.
10. Sterile graduate cylinders (25 and 50 mL).
11. Analytical balance.
12. Environmental orbital shaker incubator (28°C).
13. Spectrophotometer UV/VIS.
14. Refrigerated centrifuge.
15. Fluorescence stereomicroscope with a GFP2 filter and a HBO 100-W high-pressure mercury lamp.
16. Digital camera.

2.2.2. Stock Solutions

1. 200× Vitamin stock: 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine-HCl, 0.02 g/L thiamine HCl, 0.1 g/L calcium pantothenate, and 0.002 g/L biotin. Store at 4°C.
2. 200× Amino acid solution: 0.4 g/L glycine and 20 g/L glutamine. Store frozen in 20 mL aliquots for further use.
3. 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mM. Store at 4°C for 1 month.
4. 6-Benzyladenine (BA) 1 mM. Store at 4°C for 1 month.
5. Indole-3-acetic acid (IAA) 10 mM. Store at 4°C for 1 month.
6. Gibberellic acid (GA3) 10 mM. Filter-sterilized (22-μm pore size, Millipore) and store at 4°C for 1 month.
7. Cefotaxime (Claforan, Aventis): 300 mg/mL of stock solution, prepared by dissolving the powder in Milli-Q water, filter-sterilized (22-μm pore size, Millipore), aliquot, and stored at -20°C.
8. Kanamycin sulphate (Kan) (Duchefa): 50 mg/mL of stock solution, prepared by dissolving the powder in Milli-Q water, filter-sterilized (22-μm pore size, Millipore), aliquot, and stored at -20°C.
9. Nalidixic acid (Duchefa): 25 mg/mL of stock solution, prepared by dissolving the powder in Milli-Q water, filter-sterilized (22-μm pore size, Millipore), aliquot, and stored at -20°C.
10. Gentamicin sulphate (Duchefa): 20 mg/mL of stock solution, prepared by dissolving the powder in Milli-Q water, filter-sterilized (22-μm pore size, Millipore), aliquot, and stored at -20°C.
11. Acetosyringone (AS) (Sigma): 100 mM of stock solution, prepared by dissolving the powder in dimethyl sulfoxide (Sigma), filter-sterilized (22-μm pore size, Millipore), aliquot, and stored at -20°C.
12. For the molecular biology reagents, recipes, and preparation methods are described in Sambrook and Russell (23).

2.2.3. Media

1. Lysogeny broth medium (LB) for bacterial growth: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. Autoclave and add the appropriate antibiotics (see Note 1).
2. Liquid MS medium (LMS): MS salts (24), 0.1 g/L myo-inositol, and 30 g/L sucrose. Autoclave (see Note 2).
3. Medium for maintaining embryogenic calli (4/1.3): MS salts (24), 5 ml/L vitamins (stock solution), 5 ml/L amino acids (stock solution), 0.1 g/L casein hydrolysate, 0.1 g/L myo-inositol, 30 g/L sucrose, 4 ml/L 2,4-D (stock solution), 1.3 ml/L BA (stock solution), 8 g/L Noble agar (Difco), and pH 5.8. Autoclave and dispense in 90-mm×15-mm sterile Petri dishes.
4. Embryo differentiation medium (½ MSAC): modified MS (half strength of macroelements), 0.1 g/L myo-inositol, 0.1 g/L casein hydrolysate, 20 g/L sucrose, 2.5 g/L activated charcoal (Sigma), 8 g/L Noble agar (Difco), and pH 5.8. Autoclave, add antibiotics, and dispense in 90-mm×15-mm sterile Petri dishes (see Note 3).
5. Germination medium (25): modified MS (half strength of macroelements), 5 mL/L vitamins (stock solution), 5 mL/L amino acids (stock solution), 0.1 g/L casein hydrolysate, 0.1 g/L myo-inositol, 20 g/L sucrose, 1 mL/L IAA (stock solution), 2.5 g/L activated charcoal (Sigma), 8 g/L Noble agar (Difco), and pH 5.8. Autoclave, add 1 mL/L GA₃ (stock solution) and 300 mg/L (1 mL/L from stock solution) cefotaxime, and dispense in 90-mm×15-mm sterile Petri dishes (see Note 4).
6. Plant regeneration medium: modified MS (half strength of macroelements), 100 mg/L myo-inositol, 20 g/L sucrose, 8 g/L Noble agar (Difco), and pH 5.8. Autoclave, add 300 mg/L (1 mL/L from stock solution) cefotaxime, and dispense in 25-mm×120-mm test tubes or in 300-mL glass pots.

2.3. Bacterial Strains and Plasmid

Agrobacterium tumefaciens strains C58(pMP90) (26), a disarmed derivative of C58, and EHA105 (27), a disarmed derivative of A281, both carrying the binary plasmid pBin19-*sgfp* (28) are chosen in our protocol for calli transformation. The T-DNA of pBin19-*sgfp* contains the NOSpro-*nptII*-NOSter cassette, which is used as a selectable marker, and the 35Spro-*sgfp*-35Ster cassette, which is used as a reporter marker (29).

3. Methods

3.1. Preparation of *Agrobacterium* Cultures

1. Take one loopful of active bacteria EHA105/pBin19-*sgfp* or C58(pMP90)/pBin19-*sgfp* taken from a selection plate

cultured for 48 h at 28°C in dark and inoculate in LB with the appropriate selective antibiotics.

2. Culture overnight in an orbital shaker at 240 rpm and 28°C in dark.
3. Collect cells in sterile 50-mL conical polypropylene tubes and centrifuge at $1,574 \times g$ for 10 min. Discard the supernatant and resuspend the cells in LMS medium plus 100 μ M AS to a final optical density (OD_{600}) of 0.2 and 0.06.
4. Culture in an orbital shaker at 150 rpm for 2 h at 28°C in dark.

3.2. Preparation of Plant Cells and Infection. Embryo Selection and Plant Regeneration

1. Maintain embryogenic calli in 4/1.3 medium in dark at 25°C for 15–20 days.
2. Culture 0.08 g of embryogenic calli on $\frac{1}{2}$ MSAC medium in dark for 24 h (pre-culture) (see Note 5).
3. Add 12 mL of *Agrobacterium* suspension over embryogenic callus cultured on $\frac{1}{2}$ MSAC and incubate for 10 min. To infect embryogenic calli of Crimson Seedless, use a bacterial optical density of 0.06, and to infect Sugraone, use an OD_{600} of 0.2 (see Note 6).
4. Remove excess bacteria and incubate for 48 h in dark at 25°C for co-cultivation.
5. To eliminate *Agrobacterium*, wash with 12 mL of LMS with cefotaxime (900 mg/L) for 20 min.
6. Pick up the calli and culture in a thin layer on disks of sterile nylon meshes onto $\frac{1}{2}$ MSAC medium with cefotaxime (300 mg/L) for 10 days (see Note 7).
7. Using sterile forceps, transfer nylon meshes with cells to selective medium $\frac{1}{2}$ MSAC with kanamycin: 20 mg/L Kan for calli of Crimson Seedless or 50 mg/L Kan for calli of Sugraone. Maintain cultures at 25°C in dark and subculture to fresh medium every 3–4 weeks (see Note 8).
8. Examine periodically under a fluorescence stereomicroscope with a GFP2 filter to select GFP-positive somatic embryos.
9. Transfer individual GFP-positive somatic embryos from Kan-selective medium onto $\frac{1}{2}$ MSAC medium with cefotaxime, but without Kan. Maintain in the dark at 25°C for 20–30 days to develop.
10. Culture somatic embryos to germination medium with cefotaxime (300 mg/L) and maintain at 25°C and 16-h photoperiod with a photon flux density of 45 μ M/m²/s provided by GroLux fluorescent tubes (Sylvania).
11. Transfer germinated somatic embryos to plant regeneration medium with cefotaxime (300 mg/L) for root and shoot elongation.

12. Maintain regenerated plants on the same medium without cefotaxime in 300-mL glass pots. Transfer shoots to fresh medium every 4–6 weeks.

3.3. PCR and Southern Blot Analysis of Genomic DNA

The integration of the transgenes in the genomic DNA is usually confirmed by PCR analysis of the antibiotic selectable marker and/or the proper transgene itself.

1. Isolate genomic DNA from young leaves following the method from Lodhi et al. (30).
2. Perform PCR analysis on transgenic lines, non-transgenic controls, and negative samples with no DNA. Employ two primer pairs to detect the presence of both the *sgfp* and *nptII* genes in leaf samples from a number of regenerated transgenic plantlets. For the *sgfp* gene, use the primer pair 5' ATGGTGAGCAAGGGCGAGGA 3' and 5' GGACCATGTGATCGCGCTTC 3' that amplify a 650-bp fragment of the gene. For the *nptII* gene, use the primers 5' GACGAGGCAGCGCGGCTAT 3' and 5' AAGAAGGCGATAGAAGGCGA 3' that amplify a 600-bp fragment (29). Conduct PCR reactions in a 12 μ L volume containing 200 μ M dNTPs, 0.25 μ M of each primer, 3 mM MgCl₂, 1 $\times$ of the supplied buffer, 0.24 U of BioTaq® DNA polymerase (Bioline, London, UK), and 4 ng of the template genomic DNA. Subject reactions to 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. Detect amplified DNA by UV after electrophoresis on agarose gel (1% w/v) stained with ethidium bromide.
3. In order to confirm the stable integration of the *sgfp* gene and to determine the number of integration events in the transgenic plants, perform Southern blot analyses of genomic DNA from the transgenic lines. In brief, digest genomic DNA of the samples (20 μ g) with *Bam* HI, separated on 1% (w/v) agarose gels and blotted onto nylon membranes positively charged (Hybond®, Roche, Basel, Switzerland). Following the transfer, UV cross-link the DNA to the membrane prior to overnight hybridization with a DNA DIG-labelled probe (20 ng/mL) derived from the coding region of the *sgfp* gene and obtained after a PCR with the above described primers. Carry out detection of the hybridization signals according to the manufacturer's instructions (Roche).

3.4. Acclimatization of Plants

1. Transplant well-rooted and elongated plants into 10-cm pots with a sterilized mixture of 75% peat and 25% perlite, cover with a plastic bag, and incubate in a chamber under constant conditions: 16-h photoperiod, 27 $\pm$ 1°C for 2 weeks.
2. Raise the plastic bag gradually each day during the next 2 weeks until being completely removed.

4. Notes

1. Dispense 50 mL of autoclaved medium in 100-mL Erlenmeyer flask and add 100 μ M acetosyringone, 25 mg/L kanamycin, and the appropriate selective antibiotic: 25 mg/L nalidixic acid to growth EHA105/pBin19-*sgfp* or 20 mg/L gentamicin sulphate to growth C58(pMP90)/pBin19-*sgfp*.
2. This medium is used to resuspend *Agrobacterium* after overnight culture and infect embryogenic calli. It is also used to wash calli by adding 900 mg/L cefotaxime.
3. This medium is used without antibiotics to pre-culture the embryogenic calli before infection and to co-culture calli with *Agrobacterium*. To differentiate somatic embryos after infection, 300 mg/L (1 mL/L from stock solution) cefotaxime are added to medium after autoclaving. To select putative transgenic somatic embryos, kanamycin is also added depending on the table grape cultivar: 20 mg/L Kan (400 μ L/L from stock solution) to select calli of Crimson Seedless or 50 mg/L Kan (1 mL/L from stock solution) to select calli of Sugraone.
4. GA₃ and cefotaxime are added to media after autoclaving from filter-sterilized stock solution.
5. The use of such a low amount of embryogenic calli avoids or reduces the necrosis during the selection phase of putative transgenic somatic embryos. The pre-culture of calli on medium with activated charcoal also reduces browning.
6. Depending on the grapevine cultivar used, the concentration of *A. tumefaciens* affects the transformation efficiency, an OD₆₀₀ of 0.06 being more effective to infect Crimson Seedless calli and OD₆₀₀ of 0.2 to infect Sugraone calli.
7. Spreading the calli in a thin layer all over the surface of Petri dish was enough to reduce the necrosis and to recover transgenic somatic embryos more efficiently. Culturing the calli grouped in the centre of the Petri dish increases the necrosis and reduces the recovery of transgenic somatic embryos.
8. We have observed that optimum concentration of kanamycin used to select transformed cells depends on the cultivar, embryogenic calli of Crimson Seedless being much more sensitive to Kan than Sugraone.

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Cotton Pistil Drip Transformation Method

Tianzhen Zhang and Tianzhi Chen

Abstract

Conventional plant transformation typically includes preparation of competent plant cells or tissues, delivery of foreign genes into cells, transformed cell selection with stable incorporated foreign genes, and regeneration of transformed cells into intact plants. This process traditionally relies on tissue culture, and cotton has not been an exception to this paradigm. Though the commercialization of transgenic cotton is a resounding success, cotton transformation, which is the first step in producing transgenic cotton, is a burdensome process since there is a very long tissue culture process and a limited number of cultivars that can be regenerated. An improved process which is easier to handle and more genotype independent could efficiently generate more transgenic plants and allow meaningful analyses of gene function and transgenic plants. Cotton pistil drip by inoculating *Agrobacterium tumefaciens* onto the pistil after pollination gave rise to stable transformants. Since this transformation process in cotton occurs following pollination and during fertilization (postanthesis) but not during preanthesis as in *Arabidopsis*, the mechanism by which *Agrobacterium* enters plant cells and integrates into the cotton genome may differ from that in *Arabidopsis*. This chapter provides the detailed protocol for pistil drip, a simple *in planta* transformation method without the plant tissue culture process.

Key words: *Agrobacterium*-mediated transformation, *In planta* transformation, Cotton, Pistil drip

1. Introduction

Transgenic cotton is one of the most successful commercial genetically modified organisms, since it endows the plants with new traits which are very difficult or impossible to develop through conventional breeding. For instance, transgenic Bt cotton not only controls cotton bollworm but also may reduce its presence on other crops and may decrease the need for insecticide sprays in general (1). Compared to the commercial success of transgenic cotton, the transformation of cotton is still a difficult process. Because of the cultivar limitations and length of time in culture, many protocols

on cotton transformation adopted by different research labs are same as are based on the first two protocols of Umbeck et al. (2) and Firoozabady et al. (3). Many endeavors have been trying to improve the transformation efficiency (4, 5), to transform the favorite non-Coker cultivar (6–8), or to reduce the time in producing transgenic events by embryogenic callus transformation (9–12). However, issues such as genotype dependent, somaclonal variation, poor regeneration ability, and plant infertility still persist in the embryogenesis approach. An alternative cotton transformation through meristem organogenesis approach was developed by McCabe and Martinell (13). Transgenic cotton shoots can not only be induced in vitro from transformed meristems separated from mature seed embryos (14, 15) or cotton seedlings (16–18) but could also be screened from within intact seedlings after their meristems are subject to pricking with a sewing needle and inoculation with *Agrobacterium* (19). The advantage of meristem transformation is genotype independence, while its disadvantage is chimeric plants which potentially are not transformed in germ-line cells (13) and only 0.55% of the average transformation frequencies under a critical transformation protocol (20).

Here, we describe a novel nontissue culture-based approach for cotton transformation, pistil drip, which involves simple *in planta* inoculation of *Agrobacterium* onto cotton pistils after pollination and gave rise to stably transformed cotton during one growing season. Compared with traditional tissue culture-based transformation procedures, this method is technically easier and generally faster to obtain transgenic plants and overcomes the problems associated with somaclonal variation.

2. Materials

2.1. Plant Material

Cotton seeds of Simian 3 (*Gossypium hirsutum* L.), formerly a major cultivar in the cotton growing region of the Yangtze River Valley in China in the 1990s.

2.2. *Agrobacterium tumefaciens* Strain

Agrobacterium tumefaciens strain EHA105 harboring the binary vector pKF111(21) which contains a Basta herbicide resistance *bar* gene under the control of the CaMV 35S promoter (see Note 1).

2.3. *Agrobacterium* Culture Media

1. Luria-Bertani (LB) medium: 10 g/L of tryptone (Oxoid, Basingstoke, UK), 5 g/L of yeast extract (Oxoid), 10 g/L of sodium chloride(AR grade). Solid LB medium plus 16 g/L of agar (Plant tissue culture grade).
2. Kanamycin sulfate (Amresco, Solon, OH).
3. Rifampicin (Sigma, St. Louis, MO).

2.4. Inoculation Media

1. Sucrose (AR Grade).
2. Surfactant Silwet L-77 (Setre Chemical Company, Memphis, TN).
3. Dimethyl sulfoxide (DMSO) (Amresco).
4. Acetosyringone (Sigma): 40 mg/mL of stock dissolved in DMSO; store at 4°C for 1 month.

2.5. Other Materials

1. Paper bags fitting for flowers.
2. Plastic bags fitting for flowers.
3. Herbicide Basta containing 18.5% glufosinate (Bayer Crop Science, Japan).

3. Methods**3.1. Growth of Cotton Plants**

Grow cotton plants in field under normal field conditions and natural managements.

3.2. Preparation of *Agrobacterium* for Pistil Drip

1. Streak the *Agrobacterium tumefaciens* strain on solid LB medium supplemented with 50 mg/L of kanamycin and 20 mg/L of Rifampicin from a -80°C glycerol stock (15% glycerol in a liquid LB growth medium).
2. Incubate the plates at 28°C for 2–3 days in the dark until single colonies appear. Store the plates at 4°C for at least 1 week.
3. One day before flowering, inoculate a single colony from the recently streaked plate in 20 mL of LB supplemented with 50 mg/L of kanamycin, and grow the cells at 28°C, 150 rpm (see Note 2).
4. At 5p.m. of pollination day, pellet the cells by centrifuging for 5min at 3220×g, room temperature.
5. Discard the supernatant and resuspend the cells in 10% sucrose solution to an OD₆₀₀ of 2.0 (see Note 3).
6. Once cells are completely dispersed, add the surfactant Silwet L-77 at 0.05% (v/v) and acetosyringone at 40 mg/L of final concentration. Thoroughly mix the inoculum by gently inverting (see Note 4).

3.3. Pistil Drip Procedure

1. Self-cross flower buds in the afternoon prior to flowering (see Note 5).
2. At 5–7 p.m. on pollination day (see Note 6), drip inoculum onto stigma with a pipette, or cut stigma by hand, then drip inoculum onto the cut end of pistil (see Note 7).
3. Wrap the infected flowers with paper bags and plastic bags for 2 days (see Note 8).
4. Harvest seeds from blooming bolls.

3.4. Selection of Transformed Plants

1. Sow delinted seeds in soil.
2. Ten days after sowing, spray the plantlets with 0.05% (v/v) Basta two times at 7-day intervals (see Note 9).
3. Survived plants that show no necrosis on the leaves have a functional *bar* gene.

4. Notes

1. High virulent *Agrobacterium tumefaciens* strains are recommended in this method. Both strains EHA105 and LBA4404 work well in our practice. Other *Agrobacterium* strains used in cotton transformation based on the process of tissue culture may be suitable to this method.
2. The amount of *Agrobacterium* growing for the inoculation depends on the number of flowers to inoculate and method to inoculation. For example, if dripping pistil with a pipette (volume range 5–50 μ L), 1 mL of inoculum could drip 30–50 flowers; while dipping pistil into the inoculums, 1 mL of inoculums could only treat 10–20 flowers (see Note 7).
3. Pipette gently to resuspend the pellet with a pipette is recommended, vigorous shaking should be avoided, as it may harm the efficiency of the bacteria to transform the flowers. As discussed in floral dip in *Arabidopsis* (22), the exact cell density of the inoculum may be not so critical to *in planta* transformation since the cotton flowers have big pistils to stand the toxicity of high *Agrobacterium* density.
4. The presence of the surfactant Silwet L-77 and sucrose (or glucose) in the *Agrobacterium* inoculation media but not other factors such as *Agrobacterium* cell density, pH value, and salt ingredient of inoculation media are critical to the successful floral dip transformation in *Arabidopsis* and radish (22, 23); 5% sucrose and 0.05% Silwet L-77 are recommended in the floral dip of these two plants. Ten percentage sucrose is used in this protocol in order to decrease the bursting rate and increase the germinating of pollen grains (24) in cotton pistil, because the transformations occurred following pollination and during fertilization (postanthesis), and the inner-penetrated pollen tube, which reaches the embryo sac 13–18 h after pollination (25), may offer *Agrobacterium* a pathway to the embryo sac for subsequent transformation. The phenol compound acetosyringone could promote the transformation efficiency of *Agrobacterium* (26, 27).

5. Precautions preventing flowers from gene pollution caused by surrounding transgenic cottons through cross-pollination are required when under open conditions, especially in fields.
6. The *Agrobacterium* inoculation time is critical to transformation efficiency. Transformed seeds were not produced from inoculation at 9–11 a.m. on the first flowering day, while 0–0.06% transformation efficiency was observed when flowers were inoculated during 9–11 a.m. on the second flowering day. The most efficient time period for transformation was with *Agrobacterium* inoculation at 5–7 p.m. on the first flowering day, which resulted in a transformation efficiency of 0–0.17 and 0–0.93% in two different pistil drip treatments, respectively (see Note 7).
7. The method of *Agrobacterium* inoculation could vary according to personal preferences as long as *Agrobacterium* comes into contact with the pistil surface. Dipping the pistil directly into the inoculum for 1 s or dripping the pistil using a pipette both could produce transgenic plants in our experience. The *Agrobacterium* pistil inoculation site could affect transformation efficiency. Dripping the *Agrobacterium* inoculum directly onto the stigma at 5–7 p.m. on the pollination day produced 0–0.17% transgenic plants/number of viable seeds generated, while dripping inoculum onto the cut end of pistil after cutting the stigma by hand in the same time period resulted in an improved transformation efficiency of 0–0.93%. The pollen tube pathway hypothesis supports the higher transformation efficiency in stigma excision prior to pistil drip at 5–7 p.m. on the pollination day. Stigma excision offers *Agrobacterium* a shorter distance and a larger opening in pollen tubes, presumably increasing efficiency in accessing the interior of the style and embryo sac (ovary) and consequently more transformation events. The low transformation efficiency in pistil drip during 9–11 a.m. on the second flowering day may be the result of a shrinking pistil, as the pollen tube pathway tends to close 24 h after pollination. Therefore, the time period for transformation may be relatively short due to the limited existence of the pollen tube, unlike *Arabidopsis*, which has a longer plant development period for *Agrobacterium* mediated transformation to occur.
8. This treatment prevents the infected flowers from direct sunshine and overheating to facilitate *Agrobacterium* transformation under humid and dark conditions. In our practice, pistil drip without wrapping the infected flowers on cloudy day could also produce transgenic plants.
9. Using the *bar* gene as selective marker gene is more convenient than other selectable marker genes such as *nptII* gene using kanamycin. Transgenic cotton plantlets integrating the

bar gene could be simply sprayed with 0.05% (*v/v*) Basta one or two times after emergence and can easily be recognizable among nontransformed dead plants. Transgenic cotton plantlets could also be screened on 0.7% (*w/v*) agar containing half-strength Murashige-Skoog (MS) salt and 0.05% (*v/v*) Basta. Plantlets surviving from Basta selection in medium have open, green cotyledons and prolonged hypocotyls as well as normal root system, in contrast with nontransformed dead plantlets.

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Enhanced *Agrobacterium*-Mediated Transformation of Embryogenic Calli of Upland Cotton

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Abstract

Agrobacterium tumefaciens-mediated transformation of cotton embryogenic calli (EC) was enhanced by choosing appropriate EC and improving efficiency of coculture, selection cultivation, and plant regeneration. The binary vector pBI121 (containing a neomycin phosphotransferase II gene *npt-II* as a selection marker and a *uidA* gene as a reporter gene) was used to research transformation efficiency. After 48 h cocultivation, the number of β -glucuronidase (GUS)-positive calli characterized by yellow, loose, and fine-grained EC was twofold greater than that of gray, brown, and coarse granule EC. It indicated that the efficiency of transient transformation was affected by EC morphology. Transient transformation efficiency also was improved by cocultivation on the medium by adding 50 mg/L acetosyringone at 19°C for 48 h. Subculturing EC on the selection medium with low cell density increased the production of kanamycin-resistant (Km-R) calli lines. From an original 0.3 g EC, an average of 20 Km-R calli lines were obtained from a selection dish, and the GUS-positive rate of Km-R clones was 81.97%. A large number of normal plants were rapidly regenerated on the differentiation medium with dehydration treatments, and the GUS-positive rate of regeneration plants was about 72.6%. Polymerase chain reaction analysis of GUS-positive plantlets revealed a 100% positive detection rate for neomycin phosphotransferase II gene and *gus* gene. Southern blot of transgenic plants regenerated from different Km-R calli lines demonstrated that the target gene, mostly with the low copy number, was integrated into the cotton genome.

Key words: Cotton (*Gossypium hirsutum* L.), Embryogenic calli, Transformation, Regeneration, Tissue culture

1. Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most important fiber crops that could benefit greatly from genetic engineering. There are various cotton transformation protocols, such as transformation using shoot apices, cell suspension via a gene gun (1–5), pollen tube pathway methodology (6), and *Agrobacterium*-mediated transformation. Conventionally, *Agrobacterium*-mediated transformation

employing hypocotyls or cotyledons as explants through somatic embryogenesis has been the preferred method for cotton genetic transformation (7–12). But the protocol has hindered further development of transgenic cotton due to genotype-dependent methodologies, long transformation periods, and being labor intensive. Cotton embryogenic calli (EC) could maintain rapid embryogenesis and vigorous proliferation even after 1.5–2 years of subculture. In recent years, *Agrobacterium*-mediated transformation system using cotton EC as explants was adopted by many researchers (13, 14), and various important factors influencing the system were analyzed (15, 16). Though genotype dependence was not changed, the efficiency of this system was improved dramatically by shortening the transformation cycle and reducing labor requirements of subculture. The cocultured EC were subcultured onto the selection medium as masses (14, 16, 17). This procedure could produce a mixture of transformed clones and impair effects of kanamycin (Km) selection due to lack of complete contact between EC and Km. The other problem was the difficulty of plant production because of the low rate of embryo germination and normal plantlet regeneration. Following the first selection, Leelavathi et al. (13) obtained about 75 kanamycin-resistant (Km-R) globular embryo clusters from every 2–3 g EC (i.e., per Petri dish), and only 12 plants per Petri dish were generated ultimately. EC were initiated from a single cell, and each developed cell mass can be considered as a transformation explant. In theory, a lot of transgenic plants could be obtained using a few calli if the majority of Km-R calli lines could be selected and the conversion rate of somatic embryos into plantlets was increased. In this study, the efficiency of *Agrobacterium*-mediated transformation of cotton EC was further improved by selecting appropriate media to produce optimal EC for transformation, enhancing Km selection efficiency to obtain more Km-R calli lines and performing dehydration to promote plant regeneration.

2. Materials

All media are autoclaved at 121°C for 20 min after adjusting the pH with KOH and, after the addition of the gelling agent, *Agrobacterium* Media pH 7.0, all Cotton Tissue Culture Media pH 6.5. Autoclaved media should be cooled to <60°C before adding filter-sterilized antibiotics or acetosyringone. pH should be 5.6–5.9 after sterilization. The condition of the Cotton Tissue Culture room is temperature $25 \pm 2^\circ\text{C}$, strength of illumination 3,000–5,000 lx, and photoperiod D/N = 16 h/8 h.

2.1. *Agrobacterium* Media

Solid LB. 5 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 1.5% agar, add necessary antibiotics, and pour into plates.

Liquid LB. 5 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, add necessary antibiotics.

2.2. Cotton Tissue Culture Media and Plant Growth Media

1. Cotton seeds:
Gossypium hirsutum L., cv. Simian 3.
Gossypium barbadense L., cv. Hai 7124.
2. Basic medium (MSB):
MS basal salts (18) and B₅ vitamins (19).
3. Aseptic seedling medium:
1/2 MS: 1/2 MS inorganic salt and 6.5 g/L agar
4. *Agrobacterium* resuspend medium:
MSB1: MSB, 0.1 mg/L 2.4-D, 0.1 mg/L KT, and 30 g/L glucose
5. Coculture medium:
MSB2: MSB, 0.1 mg/L 2.4-D, 0.1 mg/L KT, 30 g/L glucose, and 6.5 g/L agar.
6. Callus induction medium:
MSB3: MSB, 0.1 mg/L 2.4-D, 0.1 mg/L KT, 2.0 g/L Phytagel (Sigma, St. Louis, USA), 30 g/L glucose.
7. Embryogenic callus proliferation medium:
MSB4: MSB, 0.1 mg/L 2.4-D, 0.1 mg/L KT, 0.91 g/L MgCl₂, 2.0 g/L Phytagel, and 30 g/L glucose.
MSB5: MSB, 0.5 mg/L indole-3-butyric acid (IBA), 0.15 mg/L, 0.91 g/L MgCl₂, 2.0 g/L Phytagel, and 30 g/L glucose.
8. Resistant embryogenic callus induction medium:
MSB6: MSB, 0.1 mg/L 2.4-D, 0.1 mg/L KT, 0.91 g/L MgCl₂, 2.0 g/L Phytagel, 100 mg/L Km, 500 mg/L Cef, and 30 g/L glucose.
MSB7: MSB, 0.5 mg/L IBA, 0.15 mg/L, 0.91 g/L MgCl₂, 2.0 g/L Phytagel, 100 mg/L Km, 500 mg/L Cef, and 30 g/L glucose.
9. Resistant embryogenic callus subculture and selection medium:
MSB8: MSB, 1.9 g/L KNO₃, 0.91 g/L MgCl₂, 2.0 g/L Phytagel, 100 mg/L Km, 500 mg/L Cef, and 30 g/L glucose.
10. Embryo germination and regeneration medium:
MSB9: MSB-NH₄NO₃, 1.9 g/L KNO₃, 0.5 g/L asparagine, 1.0 g/L glutamine, 0.91 g/L MgCl₂, 2.5 g/L Gelrite, 30 g/L glucose, placed filter paper on medium, and packed culture flask with tampon (20–22).

11. Embryogenic callus induction and plantlet development medium:

MSB10: MSB, 1.9 g/L KNO_3 , 0.91 g/L MgCl_2 , 2.5 g/L Gelrite, 30 g/L glucose.

3. Methods

3.1. Culture of *Agrobacterium* with Target Gene

1. Streak the *Agrobacterium tumefaciens* LBA4404 strain harboring a binary vector with the gene of interest on antibiotic-supplemented LB plates, and incubate for 2 days at 28°C.
2. Inoculate five colonies into a 100-mL flask containing 20 mL of LB liquid medium (supplemented with appropriate antibiotics). Grow cells at 28°C for 16–24 h on a shaker (200 rpm). Its OD at 600 nm should be 2.0 ± 0.5 .
3. Transfer bacterial cultures into a 50 mL centrifuge tube. Centrifuge at $2,057 \times g$ for 10 min. Remove supernatant and resuspend and dilute the bacterial pellet to OD₆₀₀ at 0.3–0.5 with MSB1. Be sure to break up bacterial clumps by vortexing. Transfer the dilution to a 200 mL flask and culture cells at 28°C for approx. 20 min on a shaker (200 rpm) for cocultivation.

3.2. Aseptic Seed Germination

1. Delint cotton seeds with concentrated sulfuric acid and wash three to four times with water.
2. Surface-sterilize seeds with 70% ethanol, subsequently sterilize for 3–4 h with 30% (w/v) hydrogen peroxide (H_2O_2), and then wash two to three times with sterilized distilled water.
3. Submerge the sterile seeds in sterilized distilled water for 18–24 h at 28°C then decoat and inoculate in half-strength MS medium for germination in the dark at 28°C for 3 days, and transfer to normal light culture at 28°C for 3 days.

3.3. Cotton Tissue Culture for Embryogenic Callus

1. Cut hypocotyls from aseptic seedlings into 5–7 mm segments and inoculate in MSB3 for calli induction for 40–60 days.
2. Subculture the calli on MSB10 for proliferation and somatic embryogenesis every 30 days. After two to three subcultures, embryogenesis will occur.
3. Select small EC and subculture onto MSB4 or MSB5 for EC proliferation (see Note 1). After one to three subcultures (subcultured every 3 weeks), select 0.2–0.3 g light yellow, loose, and fine-grained EC for transformation and continue to cultivate from one 6 cm plate or 100 mL flask.

3.4. Transformation

1. Several morphological variants are evident during EC proliferation and maintenance (Fig. 1a–d) (see Note 2). Inoculate yellow or light yellow fine-grained EC with the cultured *Agrobacterium* suspension for 20 min and subsequently blot dry with sterile filter papers.
2. Disperse the infected calli and coculture on MSB1 medium placed on filter paper in the dark at 19°C for 48 h (see Notes 3 and 4).

3.5. Selection/ Proliferation

1. After cocultivation, transfer and disperse 0.3 g cocultured EC on MSB6 or MSB7 for Km-resistant EC induction medium. Some vigorous growing points will be observed in the first round of selection (Fig. 2a).
2. To eliminate the false Km-resistant EC more efficiently, subculture these regions and disperse as spots on the MSB7 or MSB8 for selection and proliferation two times (Fig. 2b) (see Note 5).
3. Many Km-R calli lines are produced after three rounds of induction and selection (Fig. 2c) (see Note 5). An average of 15–35 Km-R calli lines will be generated.

3.6. Embryo Germination and Plantlet Development

1. Subculture about 0.1 g (≤ 0.1 g) Km-R calli on desiccation MSB9 to improve embryo differentiation and germination (see Note 6).
2. About 20–50 embryos per culture flask will be obtained rapidly.
3. Following one to three rounds of subculture, plantlets with three to four leaves will regenerated (Fig. 2f). Transfer plantlets (having well-defined shoot apices and roots) onto MSB10 for further growth and root establishment.
4. Cut 3–5 cm shoot off the grown plantlets and graft onto 35–40 days old seedlings; Hai 7124 (*G. barbadense* L.) with strong root system and highly resistance to disease was selected as stocks. Grow in the greenhouse for seed (see Note 7).

4. Notes

The binary vector pBI121 (containing a neomycin phosphotransferase II gene (*npt-II*) as a selection marker and a *gus* gene as a reporter gene) (23) was used to research transformation efficiency. The results are as follows:

1. Transient transformation is significantly affected by EC morphologies. The transformation efficiency of yellow or light yellow

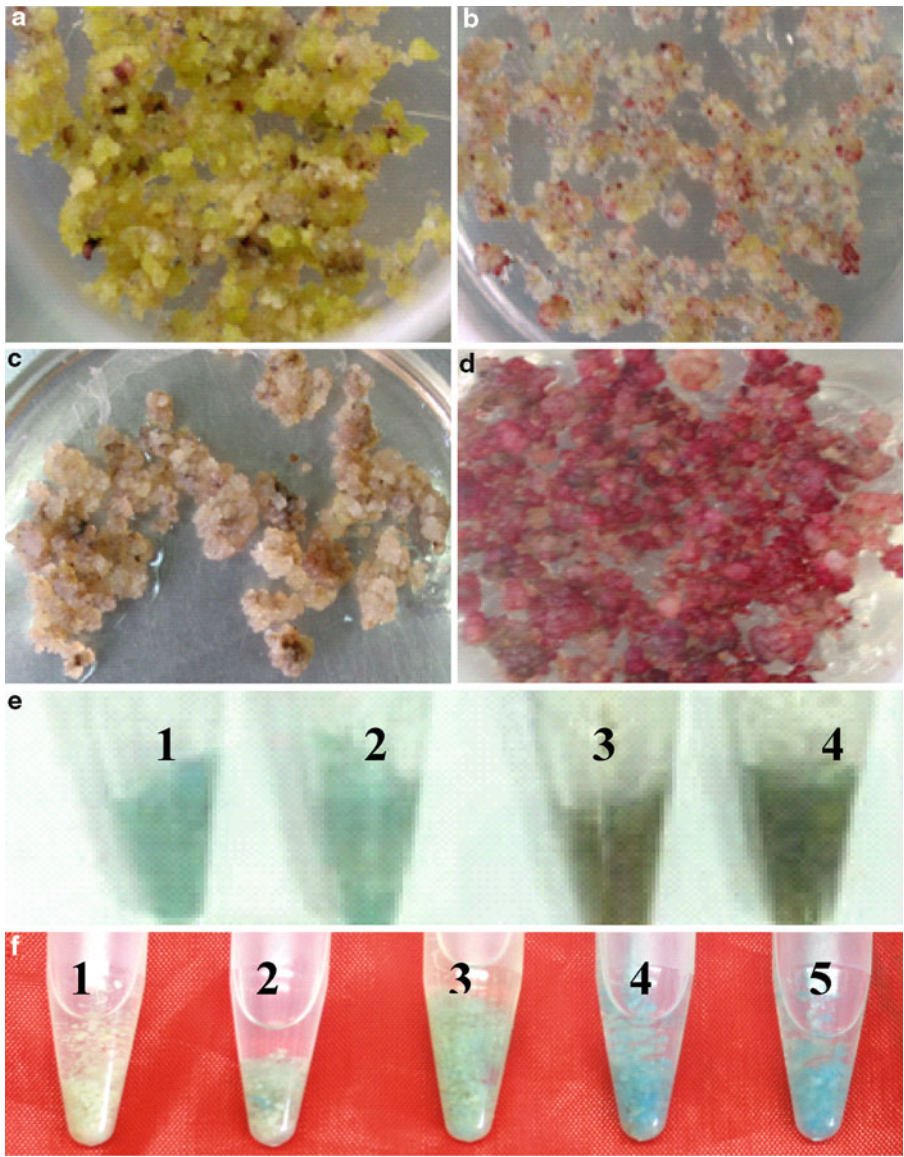


Fig. 1. The effect of EC morphologies on transient gene expression analyzed by GUS assay after transformation with vector pBI121 containing the *gus* gene. (a) Yellow loose, fine grainy EC. (b) Light yellow loose, fine grainy EC. (c) Gray coarse granule EC. (d) Brown coarse granule EC. (e) Transient transformation of four kinds of calli analyzed by GUS assay; (e1), (e2), (e3), and (e4) correspond to EC morphology (a), (b), (c), and (d). (f) GUS analysis of calli (a) cocultured for 6, 12, 24, 48, and 72 h, respectively.

fine-grained EC (Fig. 1a, b, e (1, 2)) is remarkably higher than that of gray or brown coarse-granular EC (Fig. 1c, d, e (3, 4)).

2. Light yellow, loose, and fine-grained EC can be obtained and will maintain this morphology on the MSB4 and MSB5 with some phytohormones.

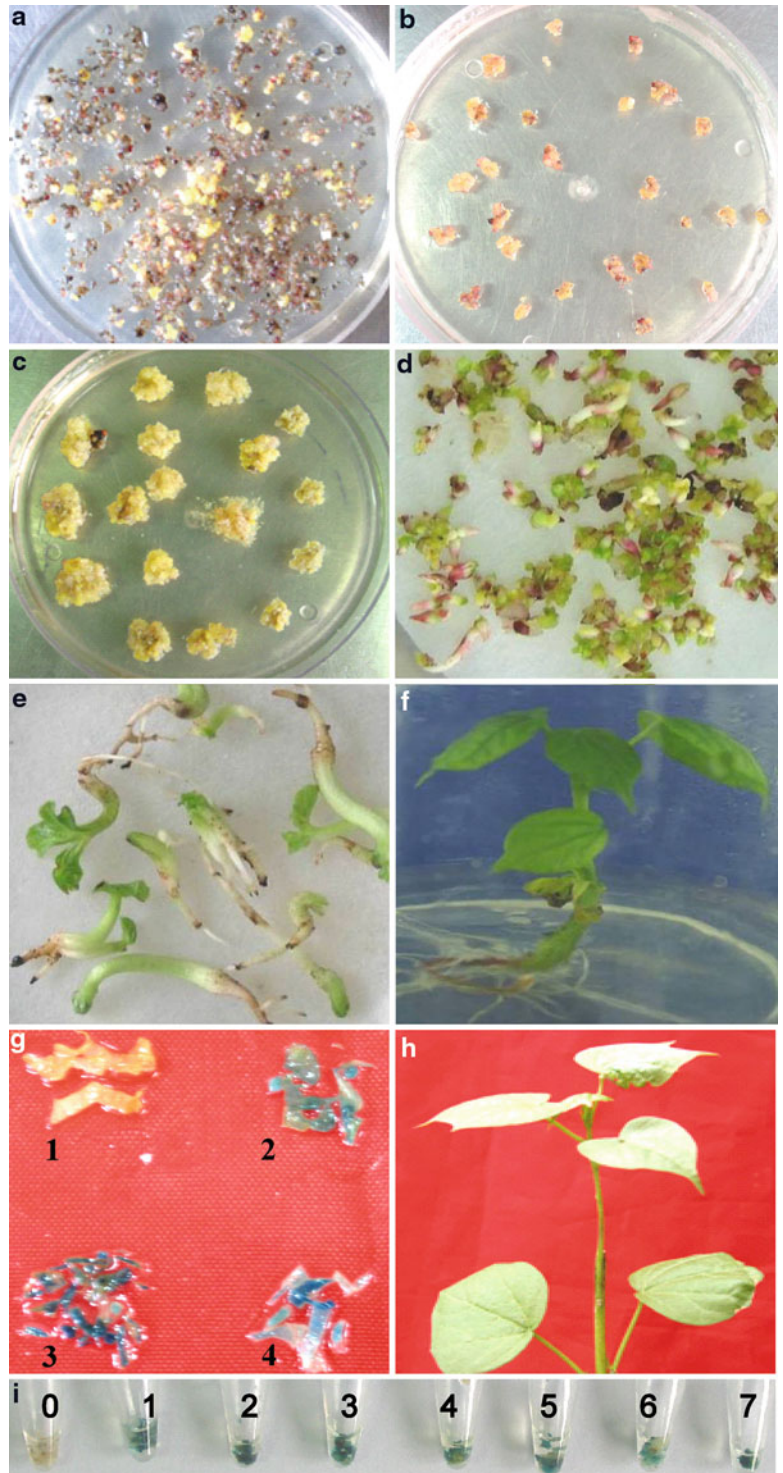


Fig. 2. Selection of Km-R calli lines and plant regeneration after transformation with vector pBI121 containing the *gus* gene. (a) Germination of Km-R calli points after the first selection. (b) Selected out Km-R calli points on the same medium. (c) Km-R calli lines after the three rounds of selection culture. (d) A mass of embryos. (e) Root regeneration. (f) Plant regeneration, (g) GUS analysis of regenerated plantlets (1: leaves of nontransformed plant, 2–4: leaves of GUS-positive plants). (h) Transgenic plantlet grafted onto a non-transformed plant. (i) GUS analysis of Km-R calli lines (0: nontransformed EC, 1–7: GUS-positive Km-R calli lines).

3. The transient transformation efficiency is at the highest level using cocultivation medium containing 50 mg/L AS and culture at 19°C.
4. After cocultivation, dispersed cocultured EC on the selection medium can be obtained. This induces the maximum number of possible transformed events (some vigorously growing sectors) in the first round of selection.
5. These vigorous sectors are dispersed by subculture on the selection medium two times as small pieces (Fig. 2b). This process eliminates the false Km-resistant EC more efficiently; many Km-R calli lines can be produced after three rounds of selection (Fig. 2c), and the GUS-positive frequency of regenerated plants is 72.6%.
6. Dual stress treatments and inoculation of ≤ 0.15 g EC promote EC differentiation, embryo germination, and plant regeneration remarkably.
7. By this protocol, we can produce thousands of transgenic plants regenerated from more than 5,000 independent transformation events every year. This transformation system further reduces the labor requirements and improves the transformation efficiency, which provides an easier, faster, and more convenient platform for production of transgenic cotton and functional analysis of key cotton genes.

Acknowledgments

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Targeted Biolistics for Improved Transformation of *Impatiens balsamina*

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Abstract

A transgenesis programme has been developed for *Impatiens balsamina* that will allow elucidation of the roles played by individual genes in the flower reversion phenomenon shown by this model species. The lack of explants exhibiting adventitious shooting in *I. balsamina* hinders *Agrobacterium*-based transformation, but the multiple shoots that arise from cotyledonary nodes present a suitable target for biolistics. These tissues can be disrupted by the helium blast effect associated with conventional biolistic devices, so we have utilised modifications to the PDS 1000/He equipment originally developed for transformation of fragile insect tissues. By loading microcarriers on to a rigid, rather than flexible, macrocarrier, the blast effect is largely eliminated, and the use of a focussing nozzle allows the bombardment to be concentrated on the target tissues. This approach reduces waste of plasmid DNA and gold microcarriers and achieves transfection at lower, less disruptive helium pressures than would otherwise be necessary to efficiently penetrate below the shoot epidermis and generate heritable transgenic lines.

Key words: Biolistics, Transformation, Cotyledonary nodes, Flower reversion, *Impatiens balsamina*

1. Introduction

Flower reversion involves a switch from floral back to vegetative development, making flowering a phase in an ongoing growth pattern rather than a terminal act of the meristem. Flower reversion is linked to environmental conditions and is most often a response to conditions opposite to those that induce flowering. Research on molecular genetic mechanisms underlying plant development has identified some of the key genes involved in the transition to flowering and flower development (1). Such investigations have also revealed mutations which reduce floral maintenance or alter the

balance between vegetative and floral features of plants. *Arabidopsis* has not been found to unambiguously revert to leaf production in any of the conditions or genetic backgrounds analysed to date and so does not present a convenient model with which to elucidate the process. Clear-cut scenarios of reversion to leaf production have, however, been described in some forms of *Impatiens balsamina* (2, 3). It is proposed that a single gene controls whether *Impatiens* reverts or continues flowering when inductive conditions are removed, and it is inferred that this gene functions to control the synthesis or transport of a leaf-generated signal. In order to explore this and other fundamental questions about the nature and function of flowering, a transgenesis programme has been developed to evaluate the functionality of transgenes that have potential to influence flower reversion in *I. balsamina*.

Extensive growth regulator trials with all conventional explant types have failed to produce an adventitious shooting regime in *I. balsamina* adequate for use with an *Agrobacterium*-based transformation approach. It was noted however, as is the case in pigeon pea (4) and *Litchi chinensis* (5), that *I. balsamina* cotyledonary nodes sampled at an early developmental stage can be induced to produce multiple shoots suitable for use with biolistic transformation. The standard Bio-Rad PDS 1000/He biolistic device we initially employed uses a helium burst in which the kinetic energy is transmitted to gold particles (microcarriers) loaded on an accelerated macrocarrier. The macrocarrier is a flexible kapton disc (45 µm thick) that is propelled by a compressed helium burst, the force of which is dictated by the value of a rupture disc. As the macrocarrier collides with a stopping screen at the end of its path, a strong residual helium flow is generated that can disrupt fragile target samples such as these immature shoots. Furthermore, the flexibility of the macrocarrier can result in poor mechanical guidance and undesirable variability in particle deposition (6). Here, we describe modifications to the PDS 1000/He which were originally developed to optimise use of the device with fragile insect tissues (7). Use of an alternative rigid macrocarrier means that the original wire-mesh stopping screen can be replaced with a smooth stopping ring. The collision between stopping ring and macrocarrier at the end of its flight acts as valve, sealing off the helium flow thereby reducing the blast effect and causing less disruption to target explants while maintaining particle penetration and impact cloud centring.

This approach addresses the same design aims behind the Helios™ Gene Gun, but avoids the variability between trials associated with that device (8). These modifications have proved to be effective for use with *I. balsamina* explants and the transfection of a range of organised plant tissues which are not amenable to *Agrobacterium*-mediated transformation.

2. Materials

Chemicals were supplied by Sigma unless otherwise stated. All aqueous solutions should be prepared with water of at least ASTM Type III purity [e.g. reverse osmosis (RO) water].

2.1. Stock Plants, Culture Media, and Solutions

1. Standard growth conditions (SGC) for stock plant maintenance are achieved using a 24°C growth chamber with cool white fluorescent lights providing 90 $\mu\text{mol}/\text{m}^2/\text{s}$ irradiance on a 16-h photoperiod.
2. Seeds of *Impatiens balsamina* cv. Dwarf Bush Flowered are obtained from the University of Reading collections.
3. Germination medium (MS3): Murashige and Skoog (9) salts, 3% sucrose, pH 5.8, solidified with 0.8% Difco Bacto agar (BD Diagnostics, Oxford, UK). All growth media are sterilised by autoclaving at 115°C, with heat-labile selection agents filter-sterilised and incorporated once media has cooled below 60°C.
4. Plasmolysis medium (MSP): prepared as MS3 but supplemented with 0.6 M mannitol, poured in 9-cm Petri dishes (see Note 1).
5. Shoot development (SD) medium: prepared as MS3 but with 1 mg/L BAP. For transformant selection, SD medium is supplemented with either 30 mg/L hygromycin (“SDH”—for use with *hptII* plasmids) or 2 mg/L phosphinothricin (“SDP”—for use with *bar* plasmids).
6. Rooting medium: prepared as MS3 but supplemented with 1 mg/L IBA and either 30 mg/L hygromycin (RMH) or 4 mg/L phosphinothricin (RMP).
7. Bacterial LB medium: 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract, pH 7.0 plus appropriate selective antibiotics depending on plasmid.
8. TE Buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA.
9. Tissue fixation solution: 0.3% formaldehyde, 10 mM MES pH 5.6, 0.3 M mannitol.
10. GUS histochemical reagent: 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) in 50 mM NaPO_4 pH 7.0. Prepare fresh when required.

2.2. Bombardment Materials

1. 2.5 M CaCl_2 , filter-sterilised in 10 mL aliquots and kept at -20°C for up to 3 months.
2. 0.1 M spermidine free base: 1 M stock prepared from powder in sterile water and maintained at -80°C in 20 μL aliquots. Prepare the 0.1 M working solution by making a 1:10 dilution

of 1 M stock in sterile water under sterile conditions. Mix well, aliquot in 10 μ L volumes, and store immediately at -20°C for up to 1 month.

- Two plasmids were used in this study: pCAMBIA 1304 (CAMBIA, Canberra, Australia) (*hptII* gene selection and *gfp:gusA* both driven by CaMV35S promoters) or pEGAD (10) (*bar* gene selection and *gfp* both driven by CaMV35S promoters). Plasmids were maintained in *E. coli* strain DH5 α for storage and large-scale amplification.

2.3. Modifications to PDS 1000/He

Microcarrier delivery in a standard PDS 1000/He (Bio-Rad) is modified by the use of the following additional/alternative components:

- Focussing nozzles with a 7-mm bore are machined from stainless steel to the dimensions shown in Fig. 2b.
- A smooth stainless steel stopping ring (that replaces the Bio-Rad stopping screen and stopping screen support) with a 3-mm-diameter bore is placed in the well at the top of the focussing nozzle. The nozzle is screwed into the microcarrier launch assembly.
- Rigid macrocarriers (which replace the flexible Bio-Rad versions) are made from thermoplastic (DuPont, UK Ltd) cast in the brass and aluminium compression mould which is machined to the dimensions shown in Fig. 2b. When ready to bombard,

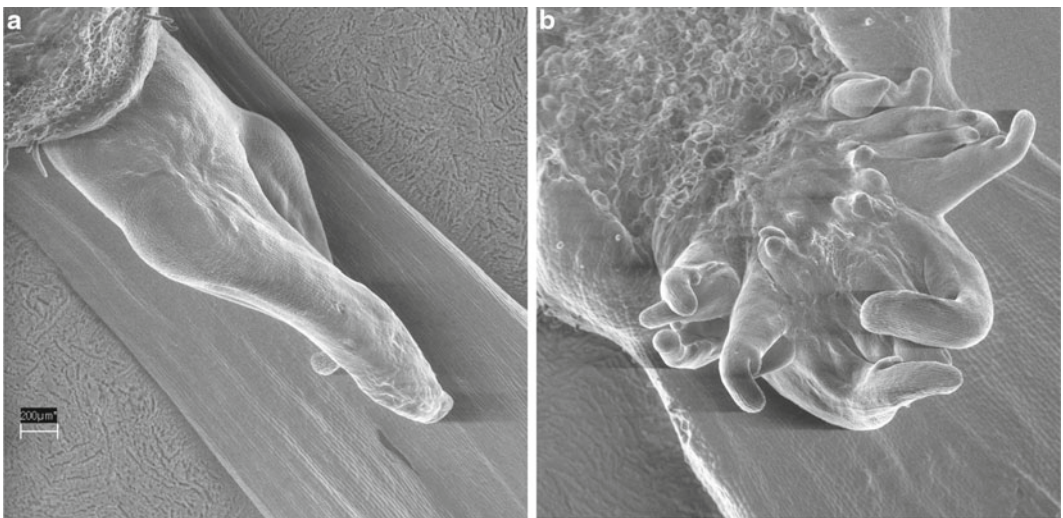


Fig. 1. Effect of *Impatiens balsamina* donor seedling age on multiple shoot emergence from cotyledonary axils. Explants from (a) 29-day-old seedling and (b) 21-day-old seedling, each shown after 4 days culture following excision. Bar = 200 μ m.

a macrocarrier is inserted (with microcarrier coated surface facing down) into a brass retaining ring and secured at the top of the launch assembly.

3. Methods

3.1. Plasmid Isolation

1. Inoculate the *E. coli* cultures (either containing pCAMBIA 1304 or pEGAD) from glycerol stocks, streak on LB medium containing 50 µg/mL ampicillin and incubate overnight at 37°C.
2. Inoculate a single colony into 10 mL ampicillin-supplemented LB medium and shake overnight at 37°C. Transfer 1 mL of this inoculum to 500 mL of ampicillin-supplemented LB medium and shake overnight at 37°C.
3. Centrifuge (3,000*g*, 10 min) 250 mL aliquots of the overnight culture to pellet the cells.
4. Isolate supercoiled plasmid DNA from the cells using a Qiagen Plasmid Maxi Kit as per the manufacturer's instructions (Qiagen, UK). Check the concentration of the eluted plasmid using a Nanodrop 1000 UV spectrophotometer (Thermo Scientific, Surrey, UK) and dilute with TE buffer to a concentration of 10 µg/µL.

3.2. Preparation of Target Explants

1. Store seeds of *I. balsamina* for at least 1 month at 4°C prior to germination. Agitate seeds in 70% (*v/v*) ethanol for 30 s and then in 10% (*v/v*) commercial bleach for 10 min.
2. Rinse three times in sterile RO water. This and all subsequent tissue handling steps must be performed in a laminar airflow hood.
3. Sow seeds aseptically on MS3 medium in Plantcon® plant tissue culture containers (ICN, Ohio) maintained under SGC. After 21 days (and 24 h before bombardment), cut a transverse stem section from each seedling, 5 mm above and below the cotyledon axils. Cut the stem longitudinally, remove the cotyledons 3 mm above the axils, and trim to give two 5-mm stem sections (each comprising a cotyledon axil with its associated axillary meristems) (see Note 2 and Fig. 1). Trim each explant so that the axillary meristems are exposed on the cotyledonary adaxial surface.
4. Arrange 15–20 explants on MSP inside a 12-mm circle in the centre of the Petri dish (see Note 3), seal with Nescofilm® (Azwel Inc., Osaka, Japan), and maintain under SGC until bombardment the following day.

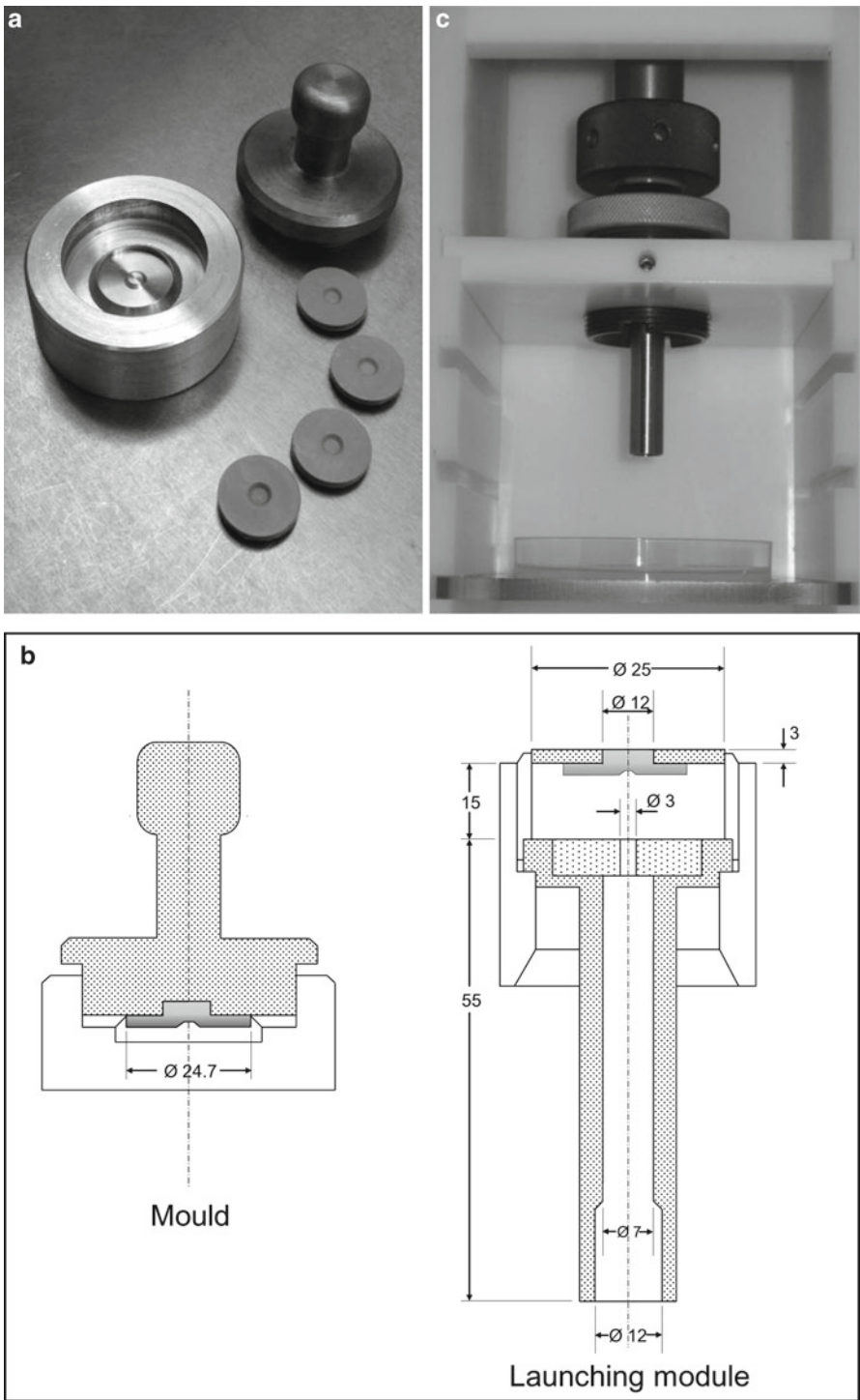


Fig. 2. Modifications to PDS 1000/He for enhanced targeting of microcarriers. (a) Rigid macrocarriers cast from thermoplastic in an aluminium mould. (b) Section through mould and launching module showing macrocarrier position (dimensions in millimetres—not to scale) (adapted from 7). (c) Modified launching module fitted in bombardment chamber.

3.3. Rigid (Thermoplastic) Macrocarrier Preparation

1. If recycling, clean the used macrocarrier by washing with 10% (v/v) commercial sodium hypochlorite bleach, rinsing with RO water, and drying with 100% ethanol (see Note 4). Alternatively, begin with a new 1.5 g sample of thermoplastic pellets.
2. Soften either the macrocarrier to be recycled or fresh thermoplastic pellets by heating in boiling water in a stainless steel spoon for approximately 30 s until the thermoplastic can be rolled into a ball.
3. Put the ball into the base of the mould (centrally) and flatten it with the pestle using rotating movements.
4. Immediately cool the resulting cast macrocarrier in cold water, so it can be easily removed from the mould.
5. Cut off the excess thermoplastic flashes created at the cutting edge of the mould's internal stainless steel ring. Keep these thermoplastic trimmings for future macrocarrier casting.
6. Immediately before bombardment, sterilise the macrocarriers by wiping with 100% ethanol and then leave to dry in the laminar airflow hood.

3.4. Gold Microcarrier Preparation

1. Weigh 60 mg of 1 μm gold microcarriers into a 1.5-mL Eppendorf tube.
2. Suspend the microcarriers in 1 mL of 100% ethanol. Shake and then vortex for several seconds.
3. Centrifuge for 5 s at 10,000 g using a microcentrifuge.
4. Pipette off the ethanol in one smooth motion.
5. Add 1 mL 100% ethanol.
6. Repeat steps 2–4.
7. Add 1 mL of sterile RO water and resuspend microcarriers by shaking and then vortexing.
8. Centrifuge for 5 s at 10,000 g and pipette off the supernatant (some of the particles may be lost at this point; however, this is a relatively dilute suspension).
9. Resuspend particles in 1 mL of sterile RO water again.
10. Shake tube and dispense suspension as 50 μL aliquots in sterile 1.5-mL Eppendorf tubes (hand shake and vortex in between tubes).
11. Store the microcarrier aliquots at 4°C.

3.5. Plasmid DNA Coating onto Gold Microcarriers

1. Add 6 μL of plasmid DNA (1 $\mu\text{g}/\mu\text{L}$ stock) to a 50 μL aliquot of the gold microcarriers (pipette up and down to resuspend) and put on platform vortex at half speed.
2. Add 50 μL 2.5 M CaCl_2 and continue vortexing.

3. Add 20 μL of 0.1 M spermidine and keep vortexing.
4. Incubate the suspension on a vortex for 10 min at full speed, then pulse centrifuge at 2,500 g for 1 s, and remove as much of the supernatant as possible (see Note 5).
5. Add 250 μL of 100% ethanol to resuspend the gold microcarriers by pipetting up and down and then continue to shake on the vortex.
6. Pulse centrifuge at 2,500 g for 1 s and remove supernatant.
7. Now, minimise all sources of vibration (e.g. switch off laminar airflow fan), add 70 μL 100% ethanol to the microcarriers, and pipette up and down to resuspend.
8. Load 10 μL of the microcarrier suspension into each macrocarrier well and allow the ethanol to evaporate (this process is aided by sitting the macrocarriers in a desiccant-filled Petri dish, e.g. CaCl_2 granules) (see Note 6). Using a stereomicroscope, check the macrocarriers for aggregates of gold particles and use the least aggregated for bombardment.

3.6. Tissue Bombardment

1. The day before the bombardment, sterilise by autoclaving: (1) the rupture disc retaining cap, (2) the macrocarrier holder (brass ring), (3) nozzle, (4) stopping ring, and (5) microcarrier launch assembly. For autoclaving, wrap the individual components in aluminium foil and when sterilised, dry thoroughly in an 80°C oven before use. Clean the insides of the nozzles and stopping rings by swabbing with a rolled piece of paper towel soaked with 100% ethanol.
2. Set the pressure on the helium tank regulator to 1,380 kPa (200 psi) in excess of the desired rupture pressure [i.e. 8,963 kPa (1,300 psi) for *Impatiens*] (see Note 7).
3. Load a 7,584 kPa (1,100 psi) rupture disc into the sterile retaining cap. Handle all rupture discs with sterile forceps (as grease, fingerprints, or even powder from plastic gloves on the rupture disc may prevent a tight seal from forming within the retaining cap). Immediately before loading, dip the rupture disc in isopropanol (do not soak the disc for an extended period of time or the disc may delaminate). Loading the rupture disc while wet forms a liquid gasket when the cap is screwed onto the gas acceleration tube, thus minimising failure of the rupture discs.
4. Secure the retaining cap to the end of the gas acceleration tube inside the bombardment chamber and tighten with the torque wrench.
5. Load the macrocarrier and stopping disc into the microcarrier launch assembly and slide this into the uppermost position in the bombardment chamber.

6. Adjust bombardment parameters for gap distance between rupture disc retaining cap and microcarrier launch assembly, i.e. 6 mm for *Impatiens* (see Note 8).
7. Place the target shelf at the desired level (9 cm for *Impatiens*) inside the bombardment chamber. Place the open Petri dish of target samples on the target shelf. Close and latch the chamber door.
8. Evacuate the chamber, hold vacuum at 84 kPa, bombard the explants, and then immediately release the vacuum (see Note 9).
9. Remove the target Petri dish from the chamber and transfer bombarded explants to an SD plate, distributing them evenly across the media. Seal the plate with Nescofilm and transfer to SGC.
10. Unload the used macrocarrier from the microcarrier launch assembly and the spent rupture disc and replace both ready for the next bombardment (see Note 10). Swab the chamber and the inside of the focussing nozzle with ethanol and replace the microcarrier launch assembly and retaining cap. After a series of bombardments, wipe the interior of the bombardment chamber with sodium hypochlorite (5% *v/v* commercial bleach), rinse with RO water, and dry with 100% ethanol.

3.7. Screening for Transformants

1. To optimise the biolistic procedure, screen for transient expression of GFP by examining a set of explants 24-h post-bombardment using an epifluorescence inverted microscope (e.g. Zeiss Axiovert 40 fitted with a 480 ± 30 nm excitation filter and a 515-nm long-pass emission filter).
2. Forty-eight hours after bombardment, conducting histochemical localisation of GUS in tissue sections will allow determination of microcarrier penetration depth (11). Cut sections of bombarded explants using a scalpel blade and vacuum infiltrate with fixation solution for ≤ 1 min. Incubate in fixative for 45 min, followed by three washes in 50 mM NaPO_4 pH 7.0. Immerse sections in histochemical reagent and incubate at 37°C for 30 min. After staining, clear chlorophyll by rinsing sections in 70% ethanol for 5 min then mount on slides for microscopy.
3. Seventy-two hours post-bombardment, transfer explants to selection medium (SDH or SDP according to plasmid used) and subculture at 14-day intervals for up to 8 weeks when resistant shoot cultures should have become established.
4. Transfer resistant shoot cultures to rooting medium (RMH or RMP according to plasmid used) (see Note 11). Subculture at 3-week intervals until adequate roots have developed that will allow survival when plantlets are potted in a 1:1 mixture of perlite and peat-based potting compost and weaned to the glasshouse.

4. Notes

1. By reducing vacuoles in the target cells, it is thought that the plasmolysis pretreatment increases the proportion of microcarriers that are delivered to nuclei, thereby increasing transgene expression efficiency following bombardment (12, 13).
2. The donor seedling developmental stage at which explants are removed has a profound effect on the subsequent emergence of axillary bud primordia. Seedlings sampled 21 days after germination (the earliest stage at which the primordia can be readily accessed) gave rise to an average of 13.1 axillary shoots per explant, but by the time the seedlings were 29 days old, shoot numbers had dropped to 4.2.
3. While bombarded cotyledonary surfaces (as opposed to axillary meristems) cannot give rise to transgenic regenerants and therefore represent a waste of microcarriers, explants dissected any smaller than 5 mm will tend to become necrotic. By partially overlaying one 5 mm explant with the next, the proportion of tissue containing axillary meristems exposed to the microcarrier impact cloud can be maximised.
4. The thermoplastic macrocarriers are relatively robust and, unlike the flexible versions, can be washed and repeatedly reused. When fissures do eventually appear, they can simply be remoulded.
5. A sample of this and subsequent supernatants from the washing steps can be electrophoresed on a 0.8% (*w/v*) agarose gel to look at the efficiency of DNA incorporation onto the microcarriers.
6. A threefold reduction in the quantity of gold microcarriers (and associated plasmid DNA) used per firing will still achieve at least the equivalent transfection rate seen when using the conventional Bio-Rad launching module arrangement.
7. When bombarding meristematic tissues of a species new to biolistics, an initial trial of helium pressures followed by histological examination for reporter gene expression will confirm if the microcarriers are making the penetrations beneath the tunica necessary to generate transformants that are not merely periclinal chimeras (14). Particle momentum must be sufficiently high to penetrate beneath the epidermal cells but should not have more damaging effects beyond the very localised particle impact site.
8. The first bombardment each day should be a “dry run” with no target material or microcarriers, conducted to ensure that the system is set up properly and that air has been fully evacuated from the gas pathway and replaced with helium.

9. These parameters result in consistent focussing, and because of the valve-like action of the rigid macrocarrier, only a very weak blast effect is generated even with a rupture disc value of 1,800 psi (12,410 kPa). Further modifications to the launching module have been trialled that eliminate all residual helium flow, but it appears that a minimal leakage of helium is necessary to ensure adequate particle speed and penetration (7).
10. If several plasmids are used on the same day, a new nozzle should be used for each plasmid to avoid cross contamination.
11. Following bombardment, non-transgenic “escapes” may persist during the shoot culture phase, but only genuine transformants appear to be capable of substantial root formation in the selective RMH or RMP media.

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A Protocol for Transformation of *Torenia*

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Abstract

This chapter describes an *Agrobacterium tumefaciens*-mediated transformation protocol for *torenia*, a plant that has several useful characteristics and is primarily used for ornamental and experimental purposes. Leaf segments of *torenia* were co-cultured with *A. tumefaciens* containing a vector plasmid for 7 days at 22°C under dark conditions on Murashige and Skoog (MS) medium containing 1 mg/L benzyladenine, 1 mg/L indoleacetic acid, and 100 µM acetosyringone. Subsequent culturing at 25°C under a 16-h photoperiod with fluorescent light on MS medium containing 1 mg/L benzyladenine, 300 mg/L carbenicillin, and selection agent (300 mg/L kanamycin or 20 mg/L hygromycin) allowed for transformant selection. Transgenic shoots were obtained from green compact calli after 2–3 months of culture in the selection medium. This method can achieve a transformation rate of approximately 5% (transformants/explant).

Key words: *Agrobacterium*, Ornamental plants, Transformation, *Torenia fournieri*

1. Introduction

The genus *Torenia* consists of about 40 species, and almost all of them are found in tropical and subtropical Asia and Africa (1). *Torenia* is a common horticultural name for several species of the genus *Torenia* (e.g., *T. fournieri*, *T. concolor*, *T. baillonii*, and their hybrids). Among these, *T. fournieri* is the most commercially important ornamental species. The *T. fournieri* “Crown” series (PanAmerican Seed Company, West Chicago, IL, USA) is usually used for transformation purposes. The “Summerwave” creeper series (Suntory Ltd., Osaka, Japan), which is an interspecies hybrid (*T. fournieri* × *T. concolor*), can be transformed using the same protocol (2).

Torenia is an ornamental plant mainly used in flower beds during the summer season. It is also an experimental plant with several useful characteristics, namely, ease of genetic transformation, ability to differentiate into adventitious structures, protruding embryo sac, and capacity for in vitro flowering. Genetic transformation of

torenia was first reported in 1995 (3), and it has been used in various transgenic studies since then (4). Torenia is a useful model plant for transgenic studies on ornamental plant characteristics such as the colour, shape, and longevity of flowers. In this chapter, we describe the transformation protocol for torenia.

2. Materials

2.1. Plant Materials

- 1. Use vegetatively growing in vitro plants of the *T. fournieri* “Crown” series for transformation (Fig. 1a) (see Note 1).

2.2. Vector Plasmids and Agrobacterium Strains

- 1. For transformation procedures, use binary vectors derived from pBin19 (5), with either NPTII or HPT as the selection marker gene (see Note 2). The cauliflower mosaic virus (CaMV) 35S promoter, which is a popular promoter for transgene expression in plants, works well in torenia (see Note 3).

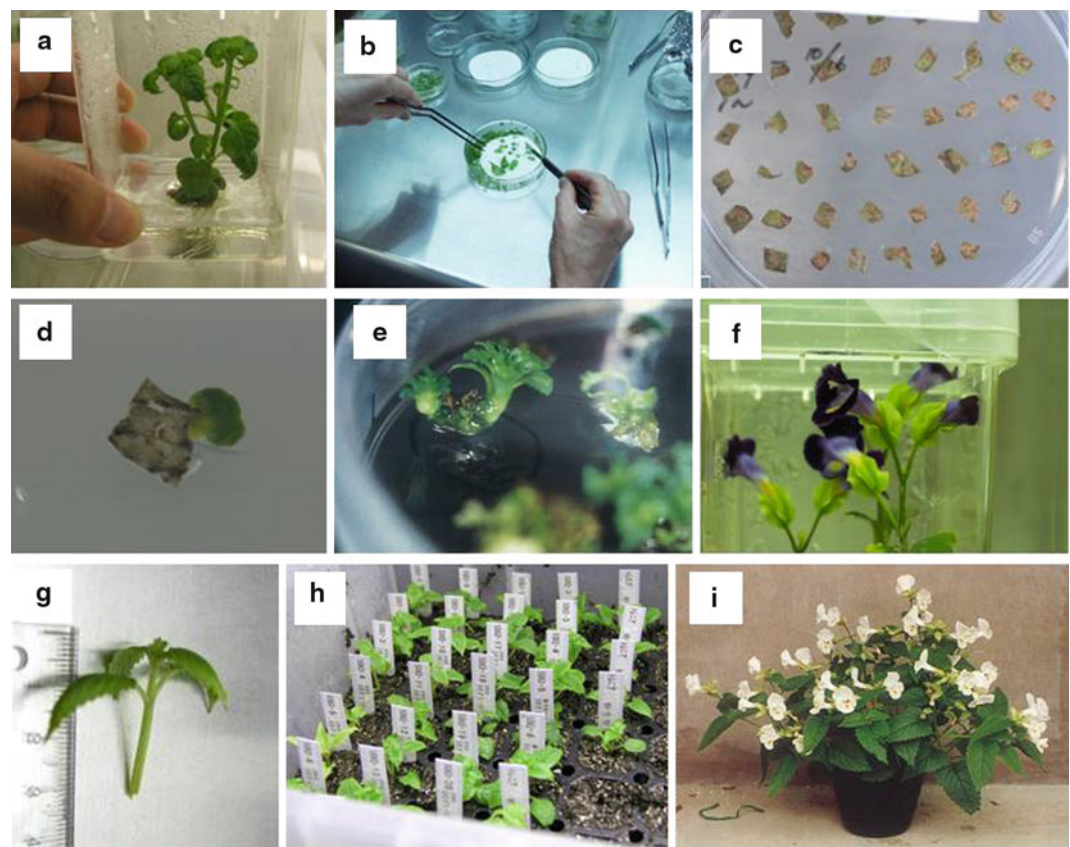


Fig. 1. Transformation of torenia. (a) A torenia plant growing in a culture box. (b) Leaves of in vitro plants cut for *Agrobacterium* infection. (c) Explants during selection culture. (d) A green compact callus. (e) Putative transformants regenerated from a green compact callus. (f) Flowering torenia in a culture box. (g) An in vitro shoot cut for acclimatization. (h) Cut shoots planted in the cells of a plug tray. (i) Flowering torenia in a greenhouse.

2. Use the *Agrobacterium* strains EHA105 (6) and AGL0 (7) for transformation (see Note 4).

2.3. Culture Medium

1. Use distilled deionized water for all experiments.
2. Conduct autoclave sterilization at 121°C for 20 min.
3. Medium can be stored at 4°C for 2 weeks.

2.3.1. Culture Medium for Plants

1. Prepare Murashige and Skoog (MS) medium (8), which is used as the basal medium for plant culture, from premixed MS salts (Murashige and Skoog Plant Salt Mixture, Nihon Pharmaceutical, Tokyo, Japan).
2. To prepare a 1,000× stock solution, dissolve the vitamins (Nacalai Tesque, Kyoto, Japan) contained in MS medium in water. Then pour 1 mL of stock solutions into 1.5-mL microtubes and store at −20°C.
3. Prepare a stock solution containing 100 mg/L benzyladenine (BA) (Wako Pure Chemical, Osaka, Japan) and 100 mg/L indoleacetic acid (IAA) (Wako Pure Chemical) and store at 4°C.
4. Dissolve acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone; Sigma-Aldrich, USA) in dimethyl sulfoxide (Nacalai Tesque) to prepare a 1 M stock solution and then store in aliquots of 0.1 mL (at a final concentration of 100 μM in 1 L medium) at −20°C.
5. Dissolve carbenicillin (100 mg/L; Nacalai Tesque) and kanamycin (100 mg/L; Nacalai Tesque) in water and store in aliquots of 10 mL at −20°C. Store hygromycin (Sigma-Aldrich; supplied in liquid form) at 4°C.
6. Dissolve the MS salts in water and add the vitamin stock solution, 30 g/L sucrose, and phytohormones. Adjust the pH to 5.7–5.8. Dispense each 50 mL of medium into a plant box containing gelling agent (a reusable plastic jar; CUL-JAR300, Asahi Glass, Science Products Dept., Tokyo, Japan) before autoclaving. After autoclaving, add the required antibiotic(s) and mix the culture medium well. For the medium used in culture dishes, add gelling agent before autoclaving, and add acetosyringone or the required antibiotics after autoclaving and mix well; following this, dispense 25 mL medium into 90-mm plastic tissue culture dishes (Kanto Chemical, Tokyo, Japan).

2.3.2. Culture Medium for Plant Materials

1. Use 1/2 MS medium (salt and vitamin concentrations at half strength) solidified with 0.2% gellan gum (Wako Pure Chemical) without any phytohormone in the plant box.

2.3.3. Medium for Co-culture with *Agrobacterium*

1. Use MS medium solidified with 0.2% gellan gum (Wako Pure Chemical) containing 1 mg/L BA, 1 mg/L IAA, and 100 μM acetosyringone to culture dishes.

2. After cooling, place a filter paper on the medium to prevent excess growth of *Agrobacterium* during co-culture in culture dishes.
- 2.3.4. Medium for Selection of Transformants**
1. Use MS medium solidified with 0.8% agar (Wako Pure Chemical) containing 1 mg/L BA, 300 mg/L carbenicillin, and a selective agent (300 mg/L kanamycin or 20 mg/L hygromycin) to culture dishes (see Note 5).
- 2.3.5. Medium for Shoot Elongation of Putative Transformants**
1. Use 1/2 MS medium solidified with 0.2% gellan gum (Wako Pure Chemical) containing 300 mg/L carbenicillin to the plant box.
- 2.3.6. Medium for Leaf Tests**
1. Use MS medium solidified with 0.8% agar (Wako Pure Chemical) containing 1 mg/L BA, 300 mg/L carbenicillin, and a selective agent (300 mg/L kanamycin or 20 mg/L hygromycin) to the culture dishes (same as Subheading 2.3.4).
- 2.3.7. Medium for Maintenance of Transformants**
1. For the first 2 or 3 months after shoot regeneration, use 1/2 MS medium solidified with 0.2% gellan gum (Wako Pure Chemical) containing 300 mg/L carbenicillin in the plant box (same as Subheading 2.3.5, see Note 6). After elimination of *Agrobacterium*, add 1/2 MS medium Solidified with 0.2% gellan gum (Wako Pure Chemical) without any phytohormone to the plant box (same as Subheading 2.3.2).
- 2.3.8. Culture Medium for *Agrobacterium***
1. Use Luria–Bertani (LB) medium (Difco, USA) solidified with 1.5% agar (Wako Pure Chemical) for bacterial culture. After autoclaving, add either 50 mg/L kanamycin or 50 mg/L hygromycin to LB medium and mix well; following this, dispense 25 mL into 90-mm plastic tissue culture dishes.

3. Methods

3.1. Preparation and Maintenance of In Vitro Plants

1. Place torenia seeds on a mesh and sealed with a stapler.
2. Soak in 70% ethanol for 10 s.
3. Transfer these seeds to sodium hypochlorite solution (1% chlorine concentration) and incubate for 3 min.
4. Then wash with sterilized water for 2 × 10 min.
5. Finally, culture on 1/2 MS medium in a plant box (see Subheading 2.3.2) at 25°C under a 16-h photoperiod with fluorescent light (photon flux density 70 μmol/s/m²).
6. After the seedlings have grown, transplant into a new medium. To maintain vegetatively growing materials, transplant either lateral shoots from lower nodes or cut stems with lower nodes

to the new medium every 3–4 weeks. Usually place four shoots in every plant box (see Note 7).

3.2. Preparation of *Agrobacterium*

1. Spread *Agrobacterium* stock (10–100 μL) spread on LB medium containing the required antibiotics.
2. Then incubate the medium at 25°C under dark conditions for 3–7 days.
3. Scrape the *Agrobacterium* and suspend in water at about $\text{OD}_{550} = 0.1$ and then use for infection.

3.3. *Agrobacterium* Infection

1. Cut leaves of in vitro plants into 4- to 5-mm squares on a wet filter paper in plastic dishes (Fig. 1b, see Note 8).
2. Incubate explants for 5 min in the *Agrobacterium* suspension in a plastic dish.
3. Remove excess *Agrobacterium* suspension using sterilized filter papers and transfer the explants to the co-culture medium.

3.4. Co-culture

1. Co-culture infected explants on the medium with *Agrobacterium* (see Subheading 2.3.3) for 7 days at 22°C under dark conditions (see Note 9).

3.5. Selection Culture

1. After co-culture, transfer the explants to a new medium for selection culture (see Subheading 2.3.4) and maintain at 25°C under a 16-h photoperiod (photon flux density 70 $\mu\text{mol/s/m}^2$) (Fig. 1c). Transfer explants to a new medium every 2 weeks.
2. Calli begin to form about 1 month after infection. Remove green compact calli measuring 2–3 mm in diameter (Fig. 1d) from the explants and transfer to a new selection medium (see Note 10).
3. Green vigorous shoots regenerating from the green compact calli constitute the putative transformants (Fig. 1e). Transfer the shoots of these putative transformants to the medium for shoot elongation (see Subheading 2.3.5).

3.6. Shoot Elongation and Leaf Test

1. Allow the shoots to elongate in the elongation medium for 3–4 weeks.
2. Cut the leaves of putative transformants into 3- to 5-mm squares (see Note 11) and culture for leaf tests on a medium containing the appropriate selection chemicals (see Subheading 2.3.6) at 25°C under a 16-h photoperiod regimen (photon flux density 70 $\mu\text{mol/s/m}^2$).
3. Leaf segments of transformants maintain their green colour and form calli on the medium. In contrast, the escape segments turn white and die. The leaf test identifies the transformants by their ability to grow on resistant selection chemicals.

3.7. In Vitro Culture of Transformants

1. As mentioned previously (see Subheading 2.3.7), use 1/2 MS medium containing 300 mg/L carbenicillin in the plant box for the first 2 or 3 months after shoot regeneration to eliminate *Agrobacterium*. Following this, use 1/2 MS medium without carbenicillin (see Subheading 2.3.2). Then culture at 25°C under a 16-h photoperiod (photon flux density 70 $\mu\text{mol/s/m}^2$).
2. To maintain transformants under vegetative growth conditions, cut lateral shoots from lower nodes or cut stems with lower nodes and transplant to a new medium every 3–4 weeks. On the other hand, if blooms are desired, repeat transplantation of apical shoots performed to induce blooming (Fig. 1f, see Note 12).

3.8. Acclimatization Procedure

1. Thoroughly mix Metro-Mix 350 (Sun Gro Horticulture, Vancouver, Canada) with water until saturated.
2. Pour water-saturated Metro-Mix 350 into 128-cell plug trays. Make a 2- to 3-cm-depth guidance hole in each cell with a toothpick. Place cell trays in a container with a lid.
3. Cut vigorously growing in vitro shoots (3–4 cm long, Fig. 1g) and apply OXYBERON® (0.5% indole-3-butyric acid; Bayer CropScience) on the cut side. Then plant the shoots into the guidance hole (Fig. 1h). Apply drops of water around the planted shoots to ensure bonding with Metro-Mix (see Note 13).
4. Spray the appropriate bactericide and/or fungicide onto the planted shoots at the beginning of incubation and then once every week. Incubate shoots at 22°C under a 16-h photoperiod (photon flux density 70 $\mu\text{mol/s/m}^2$), completely cover with lids during the first week, and then gradually uncover for acclimatization.
5. After incubation for 4 weeks, transfer the rooted shoots to pots and grow in an incubator or in a greenhouse (Fig. 1i).

4. Notes

1. Most other cultivars of *T. fournieri* can be transformed with the same procedure. The “Summerwave” interspecific hybrids (*T. fournieri* × *T. concolor*) can also be transformed with the same method (2). However, *T. baillonii* with a yellow corolla might show a much lower transformation efficiency (about 0.5% frequency) with the protocol described here.
2. A combination of the *bar* gene (9) (used as a selection marker) and selection media supplemented with 2 g/L bialaphos, a herbicide used as a selective agent, can also be used to select

the *torenia* transformants (Aida R, unpublished results). Transgenic *torenia* plants were also obtained with a combination of *phosphomannose isomerase* (as a selection marker gene that encodes the enzyme phosphomannose isomerase) and the selection medium supplemented with 20 g/L mannose and 5 g/L sucrose as carbon sources (10).

3. A translational enhancer derived from the 5'-untranslated region of the tobacco *alcohol dehydrogenase* gene enhances transgene expression by 50–100 times in *torenia* (11).
4. The *Agrobacterium* strain LBA4404 can also be used for *torenia* transformation; however, its transformation efficiency is lower than that of EHA105 or AGL0.
5. The other methods of selection are described in Note 2.
6. The selection procedure is usually enough to eliminate *Agrobacterium*. Carbenicillin is added to complete elimination of *Agrobacterium*.
7. Repeated transplanting of apical shoots leads to blooming and death. Moreover, a longer transplant period (over 5 weeks) sometimes causes sudden death even during vegetative growth.
8. To avoid desiccation, only a few leaves should be cut at the same time. Veins and petioles should be included since transgenic calli tend to form at the cut edge of leaf veins or petioles.
9. A filter paper should always be placed on the medium. Dark conditions are a necessity during co-culture, or else escape shoots tend to regenerate and hardly any transgenic plants are obtained. After 7 days of co-culture, *Agrobacterium* should not be visible around the explants. Its presence indicates overgrowth, and elimination could be difficult during the next stage of selection culture.
10. Discard loosely formed calli or white calli because transgenic plants only regenerate from dark green compact calli. Shoots that directly regenerate from explants should be considered escapes and discarded.
11. A 3- to 5-mm leaf square is the correct size for the leaf test. Larger segments tend to miss escapes, and smaller segments tend to die even if they are transformed. Take care not to injure internal areas of cut leaf segments, or else even segments of transformants tend to die.
12. A longer transplant period (>5 weeks) sometimes causes sudden death of plants.
13. The lid of the container should be opened only during planting to maintain chamber humidity during this procedure.

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Efficient Modification of Floral Traits by Heavy-Ion Beam Irradiation on Transgenic *Torenia*

Norihiro Ohtsubo, Katsutomo Sasaki, Ryutaro Aida, Hiromichi Ryuto, Hiroyuki Ichida, Yoriko Hayashi, and Tomoko Abe

Abstract

While heavy-ion beam irradiation is becoming popular technology for mutation breeding in Japan, the combination with genetic manipulation makes it more convenient to create greater variation in plant phenotypes. We have succeeded in producing over 200 varieties of transgenic *torenia* (*Torenia fournieri* Lind.) from over 2,400 regenerated plants by this procedure in only 2 years. Mutant phenotypes were observed mainly in flowers and showed wide variation in colour and shape. Higher mutation rates in the transgenics compared to those in wild type indicate the synergistic effect of genetic manipulation and heavy-ion beam irradiation, which might be advantageous to create greater variation in floral traits.

Key words: GM flower, Heavy-ion beam, *Torenia*, Model plant, Anthocyanin, Mutation breeding

1. Introduction

Heavy-ion beam consists of particles that vary widely in mass, from a proton to a uranium ion and beyond and usually those are generated through particle accelerators. There are two significant differences between photons (gamma-ray and X-ray) and ions: an ion has both mass and charge, and by contrast, a photon has neither. As the result, they provide different linear energy transfer (LET); 0.2–2.0 keV/ μm for gamma-ray and X-ray, by contrast large and varied widely for ion beams, for example, 22.5 keV/ μm for C ion and 61.5 keV/ μm for Ne ion. High-LET radiation, such as a heavy-ion beam, causes more localized, dense ionization within cells than low-LET radiation. The biological effects of high-LET radiation are generally greater than those of low-LET radiation.

The biomedical use of heavy-ion beams, which began in the 1970s, was initiated as heavy particle radiotherapy in Berkeley (1). Relative biological effectiveness values were determined for heavy ions with relative high energies at large accelerator facilities in the 1980s. Plant breeding trials began in the 1990s. Forty useful varieties have been produced mainly in horticultural crops including verbena with increased inflorescences (2), chrysanthemums with altered colour (3) and reduced axillary bud formation (4), and torenias with altered petal colour (5). The advantages of heavy-ion beam mutagenesis are low dose with high survival rates, high mutation rates, and a broad spectrum of novel traits without harming other agronomical traits (6), and the resulting products are available for commercial use after a short breeding period (7).

Heavy-ion beam breeding has mainly been developed in Japan, and there are four plants available for biological irradiation beam lines at present: RIKEN RI Beam Factory (RIBF), Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), the Wakasawan Energy Research Center Multipurpose Accelerator System with Synchrotron and Tandem (W-MAST), and Heavy-Ion Medical Accelerator in Chiba (HIMAC). Globally, there are two other facilities, Institute of Modern Physics (China) and the Laboratori Nazionali del Sud (Italy). Several accelerators for medical use are in operation all over the world, and others are being constructed. However, most accelerators for medical use can provide proton beams that have relatively low LET, and the large heavy-ion accelerators are sometimes fully devoted to basic science (nuclear physics). Therefore, the number of accelerator facilities available for mutation breeding is limited.

Torenia (*Torenia fournieri* Lind.), a worldwide annual bedding plant, is also a useful material for genetic engineering (8) because of its simple and small genome ($2n=18$, 171 Mbp) (9), applicability to tissue culture and transformation (8, 10), short generation time, and small plant size. The simple structure and colour pattern of torenia flowers enable us to detect slight change in phenotype. Normal blooming under high humidity (e.g., in plant boxes) and broad-ranging photoperiod (at least 8–16 h) help reduce the space and effort needed for maintenance.

To develop a system for efficient production of commercially valuable transgenic crops, we tried a combination of genetic manipulation and heavy-ion beam irradiation to give a wide-range variation on flower colours and shapes within a short period. We applied heavy-ion beam to wild-type and genetically modified torenia (*T. fournieri* Lind. ‘Crown Violet’) plants in which petal colour and pattern had been modified by controlling two anthocyanin biosynthesis-related genes encoding chalcone synthase (CHS) and dihydroflavonol-4-reductase (DFR) (11). Ion beams of $^{12}\text{C}^{6+}$ and $^{20}\text{Ne}^{10+}$ were applied to 11,500 leaf disks of wild type and five transgenic lines, and regenerated flowering plants were then investigated for

visible phenotypes. Of the 3,241 flowered plants, 338 (10.4%) showed altered phenotypes (12).

Creating variation in floral traits is of benefit not only for consumers but also for producers because it enables them to produce many different breeds without changing cultivation conditions. In addition, some of the obtained varieties turned out to be the useful materials for basic research on plant gene function (12, 13).

2. Materials

Transgenic materials should be handled under specific regulations for GM handling in each country. In Japan, for example, a ministerial ordinance under the Cartagena protocol domestic law specifies the containment measure of this kind. Because this rule is applicable to all the stages of the procedure, it must be noted that irradiation facilities also should be compatible with the containment measure.

Because *T. fournieri* Lind. “Crown Violet” used in this study is not a pure variety, all the important lines including starting material, transgenic lines, and heavy-ion beam-irradiated mutant lines need to be maintained vegetatively by herbaceous cutting. In general, preservation of obtained mutant phenotypes over generations requires additional effort to fix the phenotype beyond genetic segregations. In the case of vegetative reproduction, the M1 phenotype is stably maintained through the repetitive herbaceous cutting. From this perspective, it seems convenient to use vegetatively reproducible and easy-cultured plants like *torenia* and *chrysanthemum* as a starting material.

Both genetic transformation and heavy-ion beam mutagenesis in this protocol are based on the adventitious bud induction from leaf disks, as shown in Fig. 1. Transgenic *torenia* flowers used for the irradiation were produced basically according to the standard protocol using *Agrobacterium* (8, 10, 14).

2.1. *Agrobacterium*-Mediated Transformation of *Torenia*

Materials listed below are also used for the confirmation of the transgene activity by the leaf screening test as well as the preparation of plant materials for irradiation

1. *Agrobacterium* strains: any disarmed laboratory strains such as LBA4404, EHA101, and AGL0 can be used.
2. Growth chambers (day length and temperature-controllable).
3. Incubators (available at temperatures of 22–37°C).
4. Safety cabinets with Bunsen burner or cinerator.

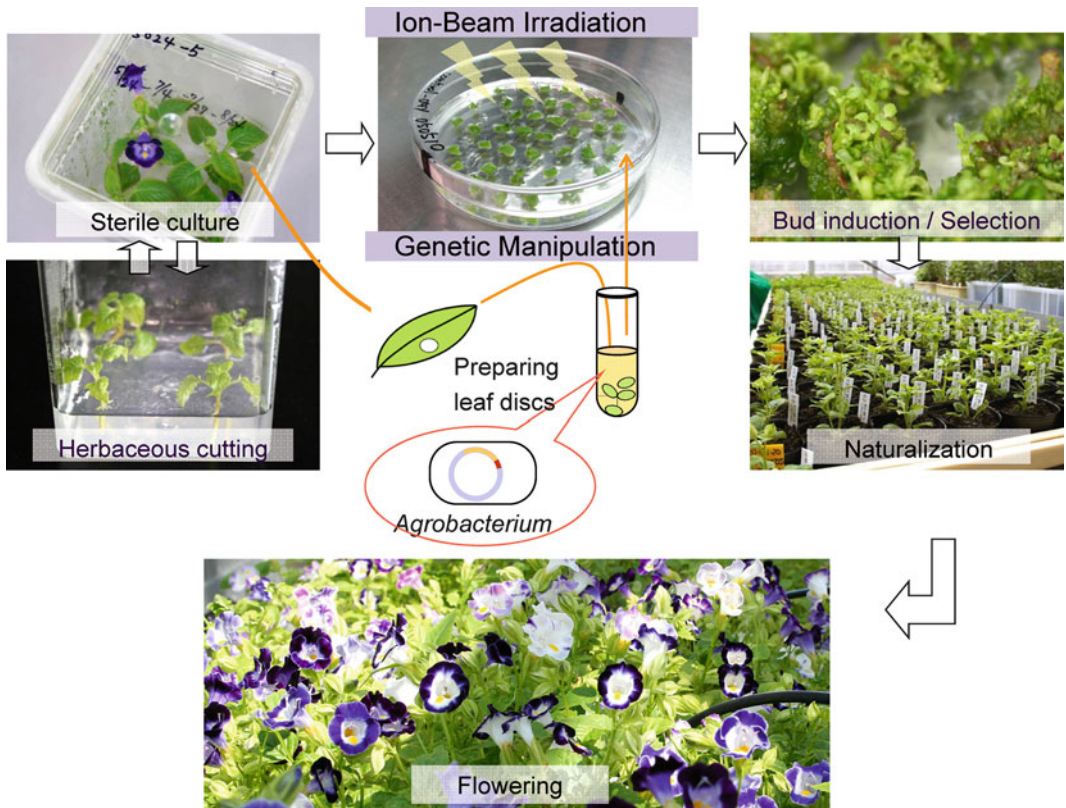


Fig. 1. Photographic representation of genetic transformation and heavy-ion beam irradiation procedures. Both genetic transformation and heavy-ion beam irradiation basically utilize the same regeneration procedure based on adventitious bud induction from leaf disks. Starting materials are aseptically propagated and maintained by herbaceous cutting. Leaf disks of 4 mm square pieces are used for *Agrobacterium* inoculation and heavy-ion beam irradiation. Required times for obtaining blooms from the starting materials are 4 months (for transgenics) and 3 months (for mutants), respectively. (Reproduced from ref. 12 with permission from the Japanese Society for Plant Cell and Molecular Biology).

5. Spreaders (HS8181 Disposable Cell Spreaders Sterile, Heathrow Scientific LLC).
6. Tweezers (K-28 Lucae's, KOWA Forceps Ind. Co., Ltd.).
7. Scalpels and disposable blades (Handle: No. 3, Blades: No. 10, FEATHER).
8. Micropipettors (Pipetman P20, P200, and P1000, Gilson) and appropriate tips.
9. Sterilized, disposable Petri dishes (CSPD90-20, 90 mm in diameter and 20 mm thick Kanto Chem. Co.).
10. Sterilized filter paper (No.2, 70 mm in diameter, Advantec).
11. Plant culture boxes with lids (CUL-JAR300, IWAKI).

12. Laboratory sealing film (Parafilm M, American National Can.).
13. 1.5-mL Microfuge tubes.
14. 70% Ethanol.
15. Antibiotics stock soln.: 100 mg/mL carbenicillin, 100 mg/mL kanamycin, 50 mg/mL hygromycin, and 2 mg/mL bialaphos (see Note 1). Prepare 50 mL and dispense in 1 mL aliquots in microfuge tube. It can be kept at -20°C up to a year.
16. LB plate, pH 7.2: Luria–Bertani (LB) medium solidified with 1.5% agar (Bacto Agar, DB).
17. 1/2 $\times$ MS plant box, pH 5.8: half-strength Murashige and Skoog (MS) medium solidified with 0.32% gellan gum (Gellan Gum, Wako). Prepare 1 L for 20 plant boxes (50 mL/box).
18. Cocultivation medium, pH 5.8: MS medium supplemented with 1 mM acetosyringone, 1 mg/L naphthalene acetic acid (NAA), 1 mg/L 6-benzylaminopurine (BA), and solidified with 0.2% (*w/v*) gellan gum.
19. Selection medium, pH 5.8: MS medium supplemented with 2 mg/L BA and solidified with 0.8% agar (Agar, Powder, Wako), 300 mg/L carbenicillin, and desired antibiotics (300 mg/L kanamycin, 20 mg/L hygromycin, or 2 mg/L bialaphos) (see Note 2).
20. Sterilized water.

2.2. Heavy-Ion Beam Irradiation

1. Heavy-ion beam irradiation facilities designed for biological materials (see Note 3).
2. 1/2 $\times$ MS plate, pH 5.8: half-strength MS medium solidified with 0.2% gellan gum (Gellan Gum, Wako). Prepare 1 L for 25 plates (40 mL/plate) (see Note 4).
3. Shoot-inducing medium, pH 5.8: MS medium supplemented with 1 mg/L BA and solidified with 0.2% gellan gum.

2.3. Regeneration and Acclimatization of Plants

Materials listed below are also used for the observation of phenotypes and evaluation of their stability through vegetative reproduction.

1. Contained green house.
2. Humidified incubator (Type 513A, Ozawa Inc.).
3. Plastic plug tray (Ando Tray 128, Ando Chem. Co.).
4. Plastic pots (TO Poly Pot, 7.5 cm in diameter, Tokai Agrisystem).
5. MetroMix 350 (Sun Glo. Horticulture Distribution Inc.).
6. Fertilized granulated soil (“Engei-Baido”, Kureha).
7. Conditioned soil: consisting of fertilized granulated soil and MetroMix mixed in equal amount.

3. Methods

One important thing for the application of this procedure to other plant species is to reduce time and effort that may be spent for the estimation of optimum irradiation dose. While irradiation system has been improved for precisely controlled irradiation, there are still many unstable factors such as plant or leaf age, tissue condition, transportation stress, and waiting time around the irradiation affecting survival rate and mutation rate unpredictably. Therefore, it is highly recommended to make a rough estimate with less effort first, then perform a wide-ranging examination to cover the variation caused by those things.

Heavy-ion beams are selectable by ion species, electrical charge, and velocity. When a high LET is required a heavier, highly charged ion is selected with a low velocity. Dose is proportional to the LET and the number of particles. The LET is selected using a range shifter. Because beam variety, beam intensity, beam line, and beam time of accelerator facilities vary by institute, each facility encourages a consultation for designing the experiment before irradiation. Biological irradiation facilities including RIBF have a fund of information on adequate irradiation condition of many kinds of plant materials. In this part, we describe the actual irradiation experiment for our research at the E5 beam line of RIBF as an example.

For biological irradiation, various factors can be configured. We used $^{12}\text{C}^{+6}$ and $^{20}\text{Ne}^{+10}$ at 135 MeV/nucleon (about 50% of light speed) for our experiments. The ranges of ^{12}C and ^{20}Ne in water are 40 and 22 mm, respectively. The important point is selecting ion species which energy is high enough to pass through the plant materials giving a uniform dose distribution. Therefore, it is highly recommended to use a facility where high-energy beam is available. RIBF is one of the good candidates from this aspect because they have high-energy accelerator facilities and a time-efficient automatic irradiation system (Figs. 2 and 3) for various biological samples. Details of available sample containers for the automatic sample changer are described in Fig. 4 and Table 1.

To avoid producing a chimeric plant, excised leaf disks should be irradiated within 2 days because adventitious shoot primordia start growing just after cutting the leaves. For the same reason, it is better to use leaf disks as irradiation material than herbaceous nodes even though chimera phenotype might be excluded by repetitive herbaceous cutting or tissue culture.

All the manipulations before acclimation should be performed aseptically in a clean bench.

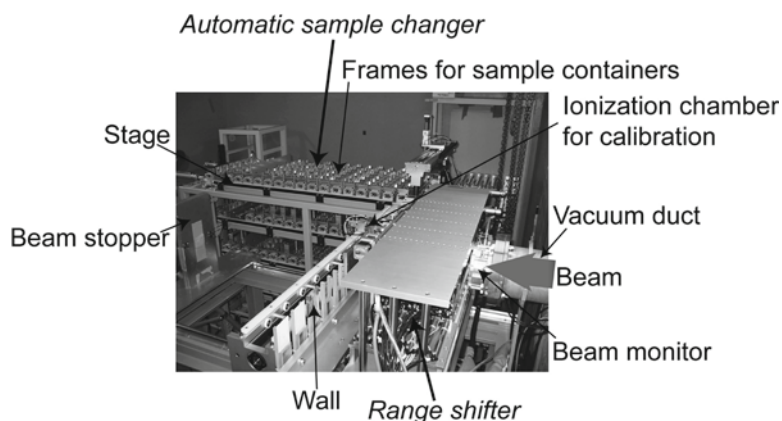


Fig. 2. The automatic irradiation system. (Reproduced from ref. 15 with permission from the Japanese Society for Plant Cell and Molecular Biology).

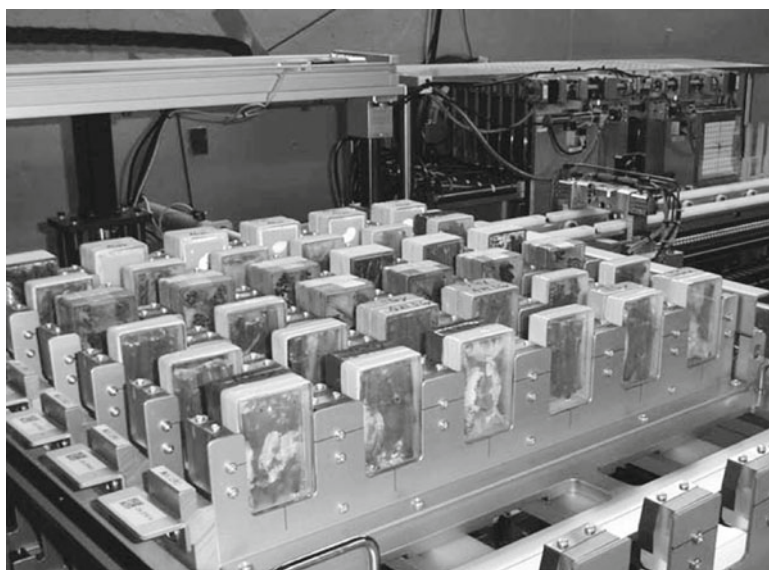


Fig. 3. Irradiation samples (Scions) in *plastic rectangular boxes* on the automatic sample changer. (Reproduced from ref. 15 with permission from the Japanese Society for Plant Cell and Molecular Biology).

3.1. *Agrobacterium*-Mediated Transformation of *Torenia*

1. Streak *Agrobacterium* containing desired gene construct onto LB plate.
2. Incubate at 25°C in darkness for 3–4 days.
3. Harvest the colonies on NA plates with sterilized water and disperse thoroughly (OD₅₅₀ = 0.1 approx.).
4. Excise torenia leaves out with 4 mm square pieces (800 pieces per inoculation) and transfer to the *Agrobacterium* suspension above (see Notes 5–7).
5. Incubate at 25°C for 5 min.

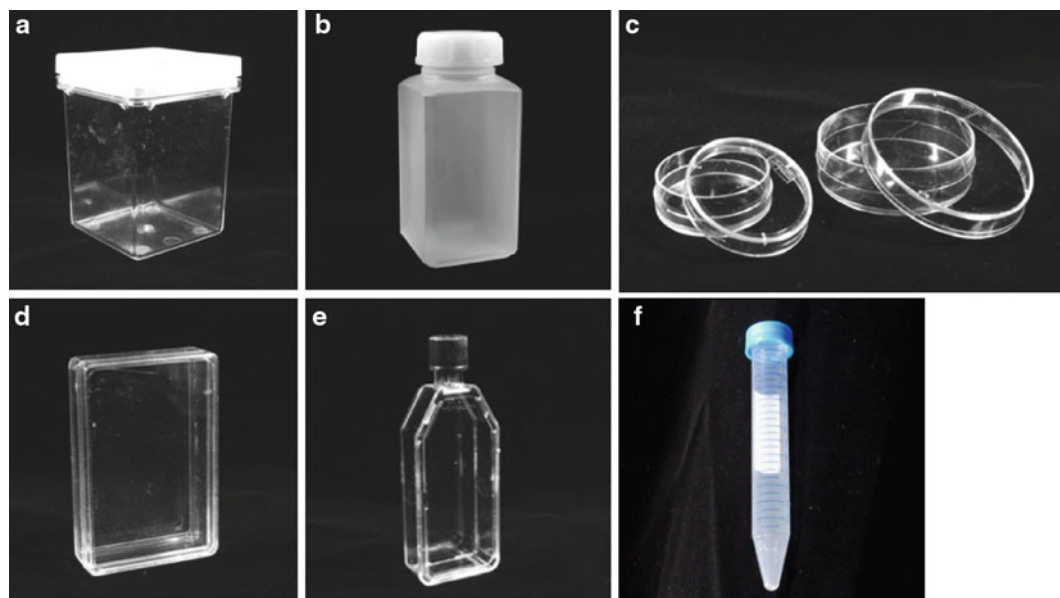


Fig. 4. Sample containers for automatic sample changer. (a) Plant box. (b) Polypropylene bottle. (c) Petri dish. (d) Plastic rectangular box. (e) Cell culture flask. (f) Centrifuge tube. (Reproduced from ref. 15 with permission from the Japanese Society for Plant Cell and Molecular Biology).

6. Remove excess *Agrobacterium* with a sterilized filter paper.
7. Put leaf disks onto cocultivation plate with a filter paper on gel top.
8. Cultivate at 25°C in darkness for a week.
9. Transfer leaf disks to selection plates containing appropriate antibiotic agent corresponding to the integrated marker gene.
10. Cultivate at 25°C under fluorescent lamp (16L/8D, 85 $\mu\text{mol}/\text{m}^2/\text{s}$).
11. Transfer leaf disks to new selection plates every 2 weeks.
12. Pick adventitious shoots on the leaf disks up and transfer to plant boxes supplemented with 1/2 MS medium by four shoots per box (see Notes 8–10).
13. Number each shoot temporary for the following leaf screening test.

3.2. Confirmation of the Transgene Activity by the Leaf Screening Test

1. After the obtained adventitious shoots grown up, excise leaf pieces of 4 mm square out and then put onto selection media containing suitable antibiotic agent corresponding to the integrated marker gene (see Note 11).
2. Cultivate at 25°C under fluorescent lamp (16L/8D, 85 $\mu\text{mol}/\text{m}^2/\text{s}$).
3. After 1–2 weeks, untransformed leaf disks sensitive to the antibiotics turn brownish. Find healthy leaf disks out and then renumber the corresponding original transformants.

Table 1
List of frames for automatic sample changer

Frame no.	Number of samples		Sample container		Size	Maker	Catalog no.	Photo
	on each frame	Type						
1xx	5	Plant box			60 mm × 60 mm × 100 mm	IWAKI CUL-JAR300	41-012-002	A
2xx	6	Polypropylene bottle			55 mm × 55 mm × 126 mm	SANPLATEC	2172	B
3xx-4xx	4 (8)	φ 90 mm Petri dish			φ 90 mm			C
5xx	6 (12)	φ 60 mm Petri dish			φ 60 mm			C
6xx	7	φ 35 mm Petri dish			φ 35 mm			
7xx	6 (12)	Plastic rectangular box			75 mm × 50 mm × 18 mm	AS ONE	1-4698-3	D
8xx-9xx	7	Cell culture flask			Culture area 24 cm ²	NUNC	152094	E
10xx	7	Micro tube, plastic bag, etc.			90 mm × 60 mm (max.)			
11xx	4				90 mm × 90 mm (max.)			
12xx	7	Centrifuge tube			15 mL	IWAKI, CORNING etc.		F

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4. Excise up to four pieces of apical bud or nodes of the transformants out from the plantlet and then put them in a plant box with 1/2 MS medium for propagation (see Note 12).
5. Perform herbaceous cutting and transfer to new plant boxes every month to maintain the lines. Excise multiple explants from one plantlet if propagation is necessary.

3.3. Preparation of Plant Materials for Irradiation

1. Propagate wild-type and transgenic torenia plants up to requisite amount by intensive herbaceous cutting. (Usually 20 plant boxes for each line are enough for a standard experiment).
2. Prepare 1/2 MS plates enough for the experimental plots and then mark each irradiation condition on the plate lid (according to the operator's instruction).
3. In a clean bench, place sterile filter paper onto disposable Petri dish and then saturate with sterile water. This is used for the preparation of leaf disks without drying.
4. Detach torenia leaves (2–3 pieces at a time) from the plantlets around 3 weeks after subculture using frame-sterilized tweezers and scalpels, then put onto the moistened filter paper above (see Note 13).
5. Cut the leaf blade into 4-mm square pieces without taking leaf vein.
6. Spread the leaf disks onto 1/2 MS plate in a single layer with adaxial side up (100 pieces per plate at max.) (see Note 14).
7. Seal the plate with laboratory film.
8. Culture at 25°C under fluorescent lamp (16L/8D, 85 $\mu\text{mol}/\text{m}^2/\text{s}$) until the delivery for irradiation.

3.4. Heavy-Ion Beam Irradiation

1. Attach the irradiation plates described in the previous section into the frames for sample containers.
2. Set the frames in the automatic sample changer (Figs. 2 and 3).
3. Apply ion beams of ^{12}C (LET 22.5 KeV/ μm , 5–80 Gy) or ^{20}Ne (LET 61.5 KeV/ μm , 5–30 Gy) automatically (see Note 15).
4. Transfer the irradiated leaf disks to MS shoot-inducing plates at 40 disks per plate.
5. Culture at 25°C under fluorescent light (16L/8D, 85 $\mu\text{mol}/\text{m}^2/\text{s}$).

3.5. Regeneration and Acclimatization of Plants

1. Correct adventitious shoots over 1 cm in height and transfer to 1/2 MS plant boxes (see Note 16).
2. Excise 3 cm of apical bud out when plantlets exceed 5 cm in height and put in the cell tray filled with MetroMix (see Note 17).
3. Acclimatize in a humidified incubator at 22°C under 80% humidity for 4 weeks.



Fig. 5. *Torenia* varieties generated by the combination of genetic manipulation and following heavy-ion beam irradiation. The original cultivar “Crown Violet” (*left panel*), five petal colour-modified transgenics (*center panel*), and the obtained mutants (*right panel*) are shown. The transgenic-based mutants showed wider variation in colour than the WT-based mutants. WT wild type, CA *CHS*-antisense, CS *CHS*-sense, DA *DFR*-antisense, DS *DFR*-sense. (Reproduced from ref. 12 with permission from the Japanese Society for Plant Cell and Molecular Biology).

4. Plantlets showing normal root formation and growth were transferred to 7.5-cm plastic pots (TO Poly Pot; Tokai Agrisystem) containing the conditioned soil.
5. Grow the plants in a contained greenhouse under natural daylight (temperature between 20 and 25°C is preferable).

3.6. Observation of Phenotypes and Evaluation of Their Stability Through Vegetative Reproduction

1. After the first ten blooms (about 30 days after potting), start observation on desired phenotypes such as petal colour, colour pattern, petal shape, corolla divergence, and pollen development (see Notes 18–20).
2. Confirm mutated phenotypes using at least ten flowers at a time.
3. Reconfirm the candidates twice using continuously maintained original plants at an interval of 1 month. Examples of obtained varieties are shown in Fig. 5.
4. Evaluate phenotypic stability in putative mutants selected by the first screening using vegetatively propagated plants for at least two different reproductions, using the observation procedure used in the first screening (see Note 21).

4. Notes

1. At least kanamycin, hygromycin, and bialaphos can be used as selection markers for *torenia* transformation.
2. Medium containing antibiotics should be prepared just before use (though it can be kept at 4°C, dark up to 1 week). Others are kept at room temperature and start using within a week.
3. The RIBF is open to the researchers in the field of nuclear physics, chemistry, and biology (15). Please refer our web page (RIKEN Nishina Center, <http://www.nishina.riken.go.jp/Eng/index.html>).
4. Dry the surface of the irradiation plates to avoid contamination during irradiation process (via water drops on plate edge).
5. Leaf disk size is important because smaller size lowers viability of leaf disks against stresses and larger size let the disks unfit to the gel surface as they grow up.
6. Do not burn the scalpels too much. It will soon make the blade dull.
7. It is convenient to use two sets of tweezers and scalpels that avoid waiting after the frame sterilization.
8. Adventitious shoots of over 3 mm in length are ready for detaching from the leaf disks.
9. Take only one shoot from a piece and then discard the rest to avoid taking genetically identical shoots originated from a single cell.
10. It is sometimes effective to take multiple shoots from a callus to reduce efforts for tissue preparation and culture. However, it requires careful attention to avoid taking shoots originated from a single cell. Up to three shoots from different edges of a leaf disk are recommended to use. If there is enough ability to maintain a quantity of tissues, take one shoot from one disk.
11. Put one leaf disk of wild-type plant per disk as a negative control.
12. If necessary, excise apical bud out and put them in a cell tray filled with moistened MetroMix for naturalization.
13. To equalize the physiological conditions of the leaf disks in each plate, prepare leaf disks from two to four independent plantlets. At least two plates should be prepared for each irradiation experiment.
14. To reduce the risk of contamination, it is recommended to reduce the number of leaf disks per plate down to 40 or less if the situation permits.

15. The optimum irradiation doses for efficient mutation, without affecting viability and/or other important traits, might be 50–80 Gy for $^{12}\text{C}^{6+}$ and 20–30 Gy for $^{20}\text{Ne}^{10+}$. Previous work in which the optimum irradiation doses for *Torenia hybrida* cv. “Summer Wave Blue” were reported to 20 Gy of $^{20}\text{Ne}^{10+}$ (5) and 50 Gy of $^{14}\text{N}^{7+}$ (16) supports this idea.
16. Up to 70 shoots were collected to obtain 50 flowering plants for each type of irradiation.
17. This is to avoid molding on MetroMix that would be caused by taking sucrose-containing gels into the soil. Extensive washing of roots enables direct implantation and rapid growth while it requires much effort.
18. Phenotypes that resulted in a reduction in plant growth or viability, such as dwarf, albino, and fasciated shoot, should be eliminated from the investigation because we thought that the lethality may impose difficulties in determining the true mutation rates for these plants. Also, the first ten blooms of each plant should be excluded from analysis because the flower phenotypes at the beginning of the flowering period are sometimes unstable, especially with regard to petal number and shape, although such instability seems a natural feature in the torenia plant (data not shown). Plants with at least one normal flower were evaluated as normal, except when the phenotype was clearly chimeric.
19. Pollen development was confirmed visually by rubbing mature anthers against petals (viability and fertility were not tested in our experiment).
20. As shown in Table 2, the average initial mutation rate in the transgenics (11.9%) is higher than that in the wild type (6.0%). While it is still arguable that the higher mutation rate in transgenics depends on the increased accessibility or variability of transgenes, it must be true that intermediate colour is more favorable than deep colour as an initial colour to distinguish subtle variations after irradiation (Fig. 5).
21. In our case, some of the obtained plants with novel traits are also utilized as a tool for basic research on the floral homeotic genes and the petal pigmentation genes. For further information, see refs. 12, 13.

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Table 2
Mutation rates of ion beam-irradiated plants after the first screening

Original lines	¹² C ⁶⁺			²⁰ Ne ¹⁰⁺		
	No. plants	Mutated	Mutation rate (%)	Original lines	No. plants	Mutated
WT	398	27	6.8	WT	420	22
CA400-29	142	19	13.4	CA400-29	252	22
CS411-3	252	35	13.9	CS411-3	255	20
CS411-7	314	30	9.6	CS411-7	290	21
DA405-2	117	22	18.8	DA405-2	247	25
DS416-20	259	40	15.4	DS416-20	295	55
¹² C ⁶⁺ subtotal	1,482	173	11.7	²⁰ Ne ¹⁰⁺ subtotal	1,759	165
WT subtotal	818	49	6.0			
TG subtotal	2,423	289	11.9			
Total	3,241	338	10.4			

Indicated as combined number and ratio of 5–80 Gy (¹²C⁶⁺) and 5–30 Gy (²⁰Ne¹⁰⁺) in each plant line. Mutant phenotype was evaluated by petal colour, colouration pattern, petal shape, corolla divergence, and male sterility. (Data for male sterility has not been included in this table.) (Reproduced from ref. 12 with permission from the Japanese Society for Plant Cell and Molecular Biology)

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Part VI

Gene Targeting, Silencing and Directed Mutation

Chapter 25

Expression of Artificial MicroRNAs in *Physcomitrella patens*

Isam Fattash, Basel Khraiwesh, M. Asif Arif, and Wolfgang Frank

Abstract

MicroRNAs (miRNAs) are ~21-nt-long small RNAs transcribed from endogenous *MIR* genes which form precursor RNAs with a characteristic hairpin structure. MiRNAs control the expression of cognate target genes by binding to reverse complementary sequences resulting in cleavage or translational inhibition of the target RNA. Artificial miRNAs (amiRNAs) can be generated by exchanging the miRNA/miRNA* sequence of endogenous *MIR* precursor genes, while maintaining the general pattern of matches and mismatches in the foldback. Thus, for functional gene analysis, amiRNAs can be designed to target any gene of interest. During the last decade, the moss *Physcomitrella patens* emerged as a model plant for functional gene analysis based on its unique ability to integrate DNA into the nuclear genome by homologous recombination which allows for the generation targeted gene knockout mutants. In addition to this, we developed a protocol to express amiRNAs in *P. patens* that has particular advantages over the generation of knockout mutants and might be used to speed up reverse genetics approaches in this model species.

Key words: RNA interference, Gene knockdown, MicroRNA, Artificial microRNA, *Physcomitrella patens*

1. Introduction

Small RNAs, 19–24 nucleotides (nt) long non-protein-coding RNAs, have been increasingly investigated. They were responsible for phenomena described as RNA interference (RNAi), cosuppression, gene silencing, or quelling (1–4). Since the discovery of RNAi in *Caenorhabditis elegans* (2), extensive studies have been performed focusing on the different aspects of RNAi. In particular, the elucidation of the essential components of RNAi pathways has extensively advanced (5, 6).

In land plants, three major classes of small RNAs, with specific sizes and dedicated functions, have evolved through a series of pathways, namely, microRNAs (miRNAs), small interfering RNAs (siRNAs), and *trans*-acting small interfering RNAs (ta-siRNAs) (7).

MiRNAs are ~21 nt non-protein-coding RNA molecules generated from genome-encoded single-stranded transcripts that form a terminal hairpin loop and a long stem where the miRNA is positioned (8, 9). These transcripts are produced by RNA polymerase II into a capped and polyadenylated pri-miRNAs (10, 11). In *Arabidopsis thaliana*, DICER-LIKE1 (DCL1) interacts with the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1) and the SERRATE (SE) protein to convert the pri-miRNA to a miRNA-miRNA* duplex (miRNA*: complementary sequence of the mature miRNA) in the nucleus (12–16). The miRNA-miRNA* duplex becomes methylated on the 2' OH of the 3' terminal nucleotides by HEN1 and is thought to be exported to the cytoplasm by HASTY, an Exportin-5 homologue (17–19). Mature miRNAs are then loaded into an RNA-induced silencing complex (RISC). The miRNA guides the RISC to cognate target RNAs that are cleaved in the middle of the complementary region between the RNA and miRNA by ARGONAUTE1 (AGO1), the catalytic component of RISC (20, 21). *Physcomitrella patens* encodes two proteins, PpDCL1a and PpDCL1b, with high similarity to the *A. thaliana* AtDCL1 and which were shown to exert distinct functions (22). PpDCL1a was shown to be the functional equivalent to the *A. thaliana* AtDCL1 required for miRNA biogenesis, whereas PpDCL1b is indispensable for miRNA-mediated target cleavage (22). siRNAs have similar structure and function as miRNAs, but they derive from long double-stranded RNA (dsRNA) of transgenes, endogenous repeat sequences, or transposons (6, 23). Transacting small interfering RNAs (ta-siRNAs), like miRNAs, are originated from nuclear-encoded TAS loci which give rise to non-coding transcripts. Processing of TAS precursors requires miRNA-directed cleavage of the transcripts, subsequent conversion into dsRNA by RNA-dependent RNA polymerases, and phased slicing of the dsRNA by DCL proteins into distinct ta-siRNAs (5, 17, 24–28). Some of the generated ta-siRNAs act like miRNAs by binding to cognate target mRNAs to control their expression at the posttranscriptional level (24, 29–31). The mode of action of small RNAs to control gene expression at the posttranscriptional level is now being developed into tools for biological research. In principle, the design of small RNA molecules that are able to target any RNA of interest in a highly specific manner allows the precise knockdown of RNAs for gene function analyses. Previous studies have shown that the alteration of several nucleotides within the miRNA/miRNA* sequence of a MIR precursor does not affect its biogenesis and maturation as long as the number of matches and mismatches in the precursor backbone, and thus the structure, is maintained (32, 33). This finding raises the possibility to modify miRNA sequences within MIR precursors according to the sequence of a selected target RNA. Thus, miRNAs can be generated artificially

that specifically target any intended RNA. This technology of artificial microRNA (amiRNA) expression successfully exploits endogenous miRNA precursors to generate small RNAs that direct gene silencing in either plants or animals (22, 34–42). Furthermore, amiRNA sequences can be easily optimized to silence one or several target transcripts without affecting the expression of other transcripts (off-targets) (43). In humans, the miR30 precursor has been modified to generate an amiRNA that downregulates target gene expression by translation inhibition (44, 45). *Arabidopsis thaliana* miRNA precursors have been modified to silence endogenous as well as exogenous target genes in the dicotyledonous plants *A. thaliana*, tomato, and tobacco (34–37, 46, 47). Gene silencing by amiRNAs in the monocotyledonous species *Oryza sativa* has been reported as well (38, 48), and recently, we established an amiRNA expression system for the moss *P. patens* (49). Gene knockdown approaches using conventional RNAi constructs rely on the expression of considerably long inverted stretches of DNA. In consequence, the expression of these constructs results in the generation of a diverse set of siRNAs that increases the possibility to affect unintended off-targets. In contrast, amiRNAs can be designed to either target a specific RNA in a highly specific manner or to target a group of sequence-related homologous RNAs. In addition, chemically induced promoters as well as tissue-specific promoters can be used to drive amiRNA expression to achieve a controlled downregulation of the intended target RNA (34, 37, 48). In this chapter, we provide protocols for the (1) design and engineering of amiRNAs, (2) the generation of amiRNA-expressing *P. patens* lines and the analysis of these lines including, (3) the analysis of amiRNA expression levels, (4) analysis of amiRNA target transcripts, and (5) detection of amiRNA target cleavage products.

2. Materials

2.1. Culture Media and Growth Conditions for *Physcomitrella patens*

1. Growth medium: 250 mg/L KH_2PO_4 , 250 mg/L KCl, 250 mg/L MgSO_4 , 1,000 mg/L $\text{Ca}(\text{NO}_3)_2$, and 12.5 mg/L FeSO_4 (pH 5.8). Solid medium contains 12 g/L agar. Sterilize by autoclaving.
2. Growth medium for protoplast isolation (reduced $\text{Ca}(\text{NO}_3)_2$): 250 mg/L KH_2PO_4 , 250 mg/L KCl, 250 mg/L MgSO_4 , 100 mg/L $\text{Ca}(\text{NO}_3)_2$, and 12.5 mg/L FeSO_4 (pH 5.8). Solid medium contains 12 g/L agar. Sterilize by autoclaving.
3. Erlenmeyer flasks containing 400 mL of suspension culture agitated on a rotary shaker at 120 rpm at 25°C under a light/dark regime of 16/8 h (Philips TLD 25, 50 $\mu\text{mol}/\text{m}^2/\text{s}^1$).

2.2. Protoplasts Isolation, Transfection, and Regeneration

1. Driselase 2% (*w/v*) (Sigma Chemical Co., Dorset, UK) in 0.5 M mannitol, filter-sterilize using a 0.22- μ M filter.
2. Sieves (Wilson, UK) of 100 and 45 μ m pore sizes.
3. 3 M medium: 5 mM MgCl_2 , 0.1% (*w/v*) 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.48 M mannitol, pH 5.6. The osmolarity of the solution should be approximately 560 mOs. Sterilize by autoclaving.

2.3. Transformation of *Physcomitrella* *patens* Protoplasts

1. Polyethylene glycol (PEG): 40% (*w/v*) PEG₄₀₀₀ in 3 M medium, pH 6.0. Filter-sterilize using a 0.22- μ m filter.
2. Linearized plasmid DNA including vector backbone or 10–15 μ g of linearized, vector-free plasmid DNA. Purify the DNA by standard ethanol precipitation and dissolve the construct at a final concentration of 0.25 μ g/ μ L in 100 μ L of 0.1 M $\text{Ca}(\text{NO}_3)_2$ (dissolve the DNA pellet in a clean bench to keep the DNA sample sterile for the protoplast transformation).
3. Autoclaved cellophane sheets.
4. Selection medium: Prepare standard solid growth medium and autoclave. Cool the medium to 60°C and add Hygromycin-B (Sigma-Aldrich) to reach the desired final antibiotic concentration (12.5 μ g/mL). Pour the medium into Petri dishes. The plates can be stored up to 4 weeks at 4°C. Hygromycin-B is used when the expression vector harbors an *hpt* expression cassette. The antibiotic depends on the selection marker cassette that is present in the expression vector of choice.
5. Regeneration medium: For 1 L, use 10 mL of each stock solution described for preparation of growth medium (KH_2PO_4 , KCl, MgSO_4 , and $\text{Ca}(\text{NO}_3)_2$), add 12.5 mg FeSO_4 , add 50 g glucose, add 30 g mannitol, adjust pH to 5.8 with KOH. Adjust the osmolarity to approximately 540 mOs using mannitol. Filter-sterilize through a 0.22- μ m filter.

2.4. One-Step Isolation of Genomic DNA from *Physcomitrella patens*

1. 10 $\times$ DNA extraction buffer: To prepare 1 L, dissolve 90.86 g Tris-HCl, 26.43 g $(\text{NH}_4)_2\text{SO}_4$, 1 mL Tween 20 in 800 mL of H_2O , adjust pH to 8.8 with HCl; before use dilute 1:10 with H_2O .
2. 96-Well PCR plate for parallel isolation of genomic DNA from 96 transformants, 96 well thermocycler.

2.5. PCR-Based Analysis

1. 10 $\times$ PCR buffer with $(\text{NH}_4)_2\text{SO}_4$: 750 mM Tris-HCl, pH 8.8, 200 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Tween 20 (Promega), 25 mM MgCl_2 (Promega), 2 mM dNTP mix (MBI Fermentas), Taq DNA Polymerase (Promega).
2. 3 mM spermidine solution: Dissolve 38.2 mg spermidine trihydrochloride (Sigma, S2501) in 50 mL of H_2O , sterilize by filtration through a 0.22- μ m filter.

3. PCR primers.
4. Thermocycler.

**2.6. Isolation
of Total RNA from
Physcomitrella patens
Using TRIzol Reagent**

1. TRIzol reagent (Invitrogen).
2. Chloroform.
3. Isopropanol.
4. 75% Ethanol in DEPC-treated H₂O.
5. RNase-free water (GIBCO).

**2.7. Real-Time PCRs
(RT-PCR)**

1. TaqMan Kit (Applied Biosystems).
2. *PpEF1α* primer pairs: forward primer 5'-CGACGCCCCCTG GACATC-3'; reverse primer 5'-CCTGCGAGGTTCCCG TAA-3'.
3. SensiMix dT Kit (Quantace).

**2.8. Small RNA
Northern Blotting
(Detection and
Analysis of amiRNAs)**

1. Polyacrylamide gel: Rotiphorese Gel 40 (37.5:1) (Roth), 10× TBE buffer (900 mM Tris–borate, 20 mM EDTA), 8.3 M urea, 10% ammonium persulfate (APS), N,N,N,N'-tetramethylethylenediamine (TEMED, pH 7.0).
2. Electrophoresis buffer: 0.5× TBE.
3. 2× RNA loading buffer: 98% deionized formamide, 2 mM EDTA, 0.01% xylene cyanol, 0.01% bromophenol blue.
4. Decade RNA Marker (Ambion), stored at –20°C.
5. Hybond N⁺ nylon membrane (GE Healthcare).
6. Whatman paper: GB002 grade.
7. Electroblotter: Trans-blot Semi-Dry Electrophoretic transfer cell (Bio-Rad).
8. UV cross-linker (Biolink BLX, Biometra).
9. DNA oligonucleotides complementary to amiRNA.
10. [γ^{32} P]-ATP: ~6,000 Ci/mmol, 10 μ Ci/ μ L.
11. T4 polynucleotide kinase (10 U/ μ L, Fermentas).
12. Nucleotide Removal Kit (Qiagen).
13. 20× SSC: 3 M NaCl, 0.3 M sodium acetate, pH 7.0.
14. Hybridization buffer: 0.05 M sodium phosphate (pH 7.2), 1 mM EDTA, 6× SSC, 1× Denhardt's, 5% SDS.
15. Washing solutions: 2× SSC, 0.2% SDS and 1× SSC, 0.1% SDS.
16. Phosphoimager (Imager FX, Bio-Rad).

**2.9. Northern Blotting
(Analysis of amiRNA
Target Transcripts)**

1. 20 μ g of total RNA.
2. RNA-denaturing buffer: 500 μ L deionized formamide, 120 μ L formaldehyde (37% w/v), 200 μ L 10× MOPS (0.2 M MOPS,

- 20 mM sodium acetate, 10 mM EDTA, DEPC-H₂O, pH 7.0), 1 μ L ethidium bromide.
3. 10 $\times$ RNA loading buffer: 50% Glycerol, 0.25% bromophenol blue, DEPC-H₂O (or RNase-free H₂O, e.g., from GIBCO).
 4. Agarose formaldehyde gel: 1.5% agarose, 1 $\times$ MOPS, 6.5% formaldehyde.
 5. RNA size marker, e.g., peqGOLD High Range RNA Ladder (PeqLab).
 6. Electrophoresis buffer: 1 $\times$ MOPS (without formaldehyde).
 7. Hybond N⁺ nylon membrane (GE Healthcare).
 8. Whatman paper: GB002 grade and GB004 grade.
 9. Turboblotter (Schleicher & Schuell) with 20 $\times$ SSC.
 10. UV cross-linker (Biolink BLX, Biometra).
 11. Hybridization buffer: 1 M Na₂HPO₄, 1 M NaH₂PO₄, 0.5 M EDTA, pH 8.0, 20% SDS, 100 μ g/mL denatured salmon sperm DNA.
 12. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
 13. [α ³²P]-dCTP-labeled DNA probe.
 14. Rediprime II Random Prime Labeling System (GE Healthcare).
 15. Washing solutions: 1 $\times$ SSC, 0.1% SDS and 0.5 $\times$ SSC, 0.1% SDS.
 16. Phosphoimager (Imager FX, Bio-Rad).
 17. Stripping buffer: 0.1% SDS heated to 100°C.

2.10. 5' RACE PCR

1. Oligotex mRNA Mini Kit (Qiagen).
2. RNA adaptor: 5'-GACUGGAGCACGAGGACACUGACAU GGACUGAAGGAGUAGAAA-3'.
3. T4 RNA ligase (10 U/ μ L, Fermentas).
4. Superscript III reverse transcriptase (200 U/ μ L, Invitrogen).
5. RNaseOUT (40 U/ μ L, Invitrogen).
6. RNase H (2 U/ μ L, Invitrogen).
7. Oligo(dT)₁₈ primer.
8. Platinum Pfx DNA Polymerase (Invitrogen).
9. 5' RACE adaptor primer: 5'-GCACGAGGACACUGACAU GGACUGA-3'.
10. 5' RACE nested adaptor primer: 5'-GGACACTGACATGGAC TGAAGGAGTA-3'.
11. Gene specific 5' RACE primer and nested primer.
12. Phenol/chloroform/isoamyl alcohol (25:24:1).
13. 3 M sodium acetate, pH 5.2.
14. 95 and 70% ethanol.

15. GlycoBlue (Applied Biosystems).
16. QIAquick gel extraction kit (Qiagen).

2.11. Electrophoretic Separation of DNA

1. 1–2% Agarose gel.
2. 50× TAE buffer: 2 M Tris–HCl, pH 8.0, 1 M glacial acetic acid, 50 mM EDTA, pH 8.0.

3. Methods

3.1. Design of amiRNAs Using the WMD Platform

An amiRNA targeting the gene of interest can be designed using the amiRNA designer interface WMD (Web MicroRNA Designer; wmd3.weigelworld.org) (6, 37). This tool has been extended to >30 species for which genome or extensive EST information is available (see table 1 in ref. 14). The designed amiRNA has to meet certain criteria, which have to be considered when choosing the final amiRNA (see Note 1):

1. The designed amiRNA contains an uridine residue at position 1 and an adenine residue at position 10, both of which are overrepresented among natural plant miRNAs and increase the efficiency of miRNA-mediated target cleavage (50).
2. The amiRNA exhibits 5' instability relative to the miRNA* which positively affects separation of both strands during RSIC loading (50, 51).
3. No mismatch between positions 2 and 12 of the amiRNA for all targets.
4. Similar mismatch pattern for all intended targets.
5. Absolute hybridization energy between –35 and –38 kcal/mol.
6. Target site position: There is no evidence that the position of the target site in the target transcript has an effect on effectiveness, but target sites in most endogenous miRNA targets are found toward the 3' end of the coding regions.

3.2. Engineering an amiRNA Precursor by Overlapping PCR

After selection of an amiRNA, the designed 21 nt amiRNA sequence has to be engineered into a miRNA precursor in order to replace the endogenous miRNA/miRNA* sequences within the precursor backbone. This is achieved by overlapping PCR using a miRNA precursor fragment that is cloned into a common cloning vector (37). To generate an amiRNA precursor that can be used for amiRNA expression in *P. patens*, we have used the *A. thaliana* miR319a precursor backbone (see Note 2). MiR319 and the corresponding *MIR319* loci are conserved between *P. patens* and *A. thaliana*, and we were able to show that amiRNAs

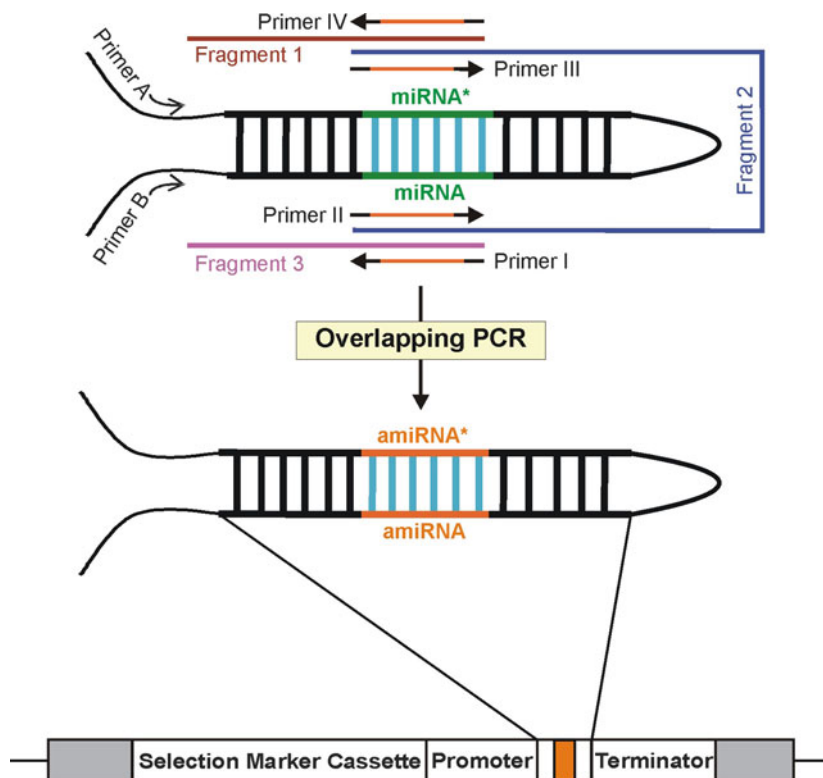


Fig. 1. Engineering an amiRNA sequence by overlapping PCR. Oligonucleotide primers I–IV are used to replace miRNA and miRNA* regions by amiRNA sequences. The plasmid pRS300 containing the *Arabidopsis thaliana* miR319a precursor is used as PCR template. First, amplify fragment 1 with primers A and IV, fragment 2 with primers II and III, and fragment 3 with primers I and B. Primers A and B are based on template plasmid sequence. In a second round of PCR, combine fragments 1, 2, and 3 as a template and perform an overlapping PCR using the primers A and B. Finally, clone the modified precursor harboring the engineered amiRNA sequence into a suitable expression vector.

are correctly processed from the *A. thaliana* miR319 precursor sequence in *P. patens* (49):

1. The WMD (Web MicroRNA Designer; www.wmd3.weigel-world.org) interface includes the “Oligo” software tool which allows automatic design of oligonucleotide primers I–IV (primer I: miRNA-sense, primer II: miRNA-antisense, primer III: miRNA*-sense, primer IV: miRNA*-antisense; Fig. 1) which are used for overlapping PCR.
2. Overlapping PCR is carried out using the plasmid pRS300 as template which harbors the *A. thaliana* MIR319a precursor cloned into the *Sma*I site of pBSK. The oligonucleotide primers A and B are based on the plasmid vector backbone sequence.
3. Amplify three individual PCR products using the primer pairs A and IV, II and III, and I and B, respectively.
4. Combine the obtained PCR products and amplify the engineered amiRNA precursor in a final PCR using the primers A and B (Fig. 1).

5. Clone the DNA fragment obtained from the overlapping PCR into common cloning vectors.
6. After cloning, confirm successful exchange of the miRNA/miRNA* sequences by sequencing.
7. For subcloning of the amiRNA precursor fragment into a suitable plant expression vector, use any sites of the pBSK multiple cloning site since they are part of the PCR fragment.
8. For the expression of amiRNAs in *P. patens*, use the vector pPCV which drives expression of the amiRNA from a CaMV 35S promoter and harbors an *hpt* selection marker cassette (52).

3.3. Transformation of *Physcomitrella patens* Protoplasts

PEG-mediated transformation of *P. patens* protoplasts was performed according to standard procedures (53):

1. Small-scale preparation of moss material: Starting from a standard *P. patens* liquid culture grown in Erlenmeyer flasks, inoculate 200 mL of growth medium for protoplast isolation (reduced $\text{Ca}(\text{NO}_3)_2$) with moss material corresponding to 10 mg dry weight.
2. Grow the culture in a 500-mL Erlenmeyer flask under standard conditions.
3. After 4 days, exchange the medium and cultivate for another 3 days. The complete culture is used for protoplast isolation. Alternatively, *P. patens* plants can be grown in a 5-L bioreactor in standard growth medium at a reduced pH of 4.5. For protoplast isolation, 100–200 mL of these cultures is used.
4. Harvest the plant material by filtration through a 100- μm protoplast sieve.
5. Transfer the material to a Petri dish (9-cm diameter) and add 8 mL of 0.5 M mannitol solution.
6. Add 8 mL of the Driselase stock solution. The final concentration of Driselase is 2% (*w/v*).
7. Seal the Petri dish with Parafilm and cover it with aluminum foil. Incubate for 45 min at room temperature on a rotary shaker.
8. Pass the moss material successively through sieves with a mesh size of 100 and 45 μm and divide the filtrate into two glass tubes.
9. Centrifuge the filtrate for 10 min at $45 \times g$ in the glass tube.
10. Discard the supernatant and wash the protoplasts by resuspending each pellet in 10 mL of 0.5 M mannitol and gentle rolling of the glass tubes between your hands.
11. Centrifuge again for 10 min at $45 \times g$, discard the supernatant, and resuspend each pellet in 5 mL of 0.5 M mannitol. Combine both samples.

12. Take a 100- μ L aliquot with a cut pipette tip and determine the protoplast number using a counting chamber.
13. Meanwhile centrifuge the combined protoplasts again for 10 min at $45\times g$. Discard the supernatant and resuspend the pellet in 3 M medium adjusting to a density of 1.2×10^6 protoplasts/mL.
14. Transfer the DNA solution into a glass tube and carefully add 250 μ L of the protoplast solution using a cut pipette tip.
15. Add 350 μ L of the PEG solution and mix gently by rolling the tube. Incubate the mixture for 30 min at room temperature and mix again every 5 min by gentle rolling.
16. Then, dilute the mixture with 3 M medium every 5 min adding 1, 2, 3, and 4 mL, respectively, and carefully mix the solution after each step by rolling the tube.
17. Centrifuge for 10 min at $45\times g$, discard the supernatant, and resuspend the protoplasts in 3 mL of regeneration medium.
18. Transfer two times 1.5 mL of the protoplast solution into two wells of a 6-well culture plate.
19. Seal the plate with Parafilm and incubate overnight in the dark at 25°C followed by incubation under normal growth conditions for 10 days. During this time, regeneration of protoplasts is initiated.
20. Plate 1 mL of the regenerating protoplasts onto a 9-cm Petri dish containing solidified standard growth medium. The medium should be covered with a sterile cellophane sheet which facilitates the transfer of the regenerating plants at subsequent stages.
21. Grow the cultures under standard conditions for 3 days.
22. To select stable transformed plants, transfer the cellophane sheet with the cultures onto solidified growth medium containing the appropriate concentration of the selection antibiotic (depending on the used expression vector) for 2 weeks, followed by a 2-week release period on medium without selection antibiotic and a second selection period of 2 weeks. Plants surviving the second round of selection are considered to be stable transformants.

3.4. PCR-Based Analysis of Putative *Physcomitrella patens* Transgenic Lines

After selection of regenerating plants, genomic DNA of individual lines can be analyzed by PCR with primers flanking the amiRNA sequence present in the expression construct to identify transgenic lines that had integrated the amiRNA construct:

1. Fill 50 μ L of $1\times$ DNA extraction buffer into each well of a 96-well PCR plate.
2. Place 1–5 mg plant material (approximately corresponding to one gametophore or 2–3 protonema filaments) from each putative transformant into one well containing 50 μ L of $1\times$ DNA extraction buffer.

3. Cover the plate using self-adhesive aluminum foil and incubate for 15 min at 45°C in a thermocycler.
4. Directly use 10 µL of the extract for PCR analysis or store it at -20°C until needed.
5. For PCR amplification mix:
 - 10 µL of moss extract.
 - 5 µL of 10× PCR buffer with (NH₄)₂SO₄.
 - 4 µL of spermidine (3 mM).
 - 6 µL of MgCl₂ (25 mM).
 - 5 µL of dNTP mix (2 mM each).
 - 1 µL of forward primer (20 pmol/µL).
 - 1 µL of reverse primer (20 pmol/µL).
 - 0.5 µL of Taq DNA Polymerase (5 U/µL, Promega).
 - 18.5 µL of H₂O.

The first denaturing step of the PCR is carried out for 5 min at 94°C. In total 40 PCR cycles are recommended. The annealing temperature and the extension time are dependent on the primer sequences and the length of the PCR product.

3.5. RNA Isolation

High-quality total RNA is required for the molecular analysis of transgenic lines including the analysis of (1) amiRNA expression, (2) amiRNA target expression, and (3) validation of target cleavage. RNA isolation from *P. patens* using TRIzol reagent (Invitrogen) results in high yields of intact RNA. Furthermore, small RNAs are nicely recovered by this method. We follow the manufacturer's instructions with minor modifications (see Note 3):

1. Homogenize moss material in liquid nitrogen with mortar and pestle and transfer to a 15-mL Falcon tube (keep the plant material frozen until you add TRIzol).
2. Add 1 mL of TRIzol to 50–100 mg frozen tissue and mix by vortexing.
3. Incubate 5 min at room temperature.
4. Centrifuge 15 min at 5,000 × *g* and 4°C.
5. Transfer the supernatant into a new Falcon tube (discard cell debris).
6. Add 0.2 mL of chloroform per initial millilitre of TRIzol, vortex for 15 s.
7. Incubate 5 min at room temperature.
8. Centrifuge 30 min at 5,000 × *g* and 4°C.
9. Pipette upper aqueous phase into a new Falcon tube and add an equal volume of isopropanol and mix.

10. Incubate 10 min at 4°C.
11. Centrifuge 20 min at 5,000×*g* and 4°C. Discard the supernatant carefully without losing the RNA pellet.
12. Wash the pellet with 1 mL of 75% ethanol per initial millilitre of TRIzol and centrifuge 8 min at 5,000×*g*, 4°C.
13. Remove the supernatant quantitatively and dry the RNA pellet.
14. Dissolve the RNA in 100–200 µL of RNase-free water. You may incubate the sample for 10 min at 65°C to dissolve the RNA.

3.6. Small RNA Blotting

To prove the correct maturation of the amiRNA from the *A. thaliana* miR319a precursor and its accumulation in the transgenic lines, you should perform small RNA gel blot analysis with an antisense probe for the amiRNA:

1. Preparation of polyacrylamide gel mix: 12 mL of Rotiphorese Gel 40 (37.5:1), 4 mL of 10× TBE buffer, 20 g urea (8.3 M), 320 µL of APS (10%), 42.4 µL of TEMED, adjust the volume to 40 mL with H₂O. We prepare 16 cm×20 cm gels using 1 mm spacers.
2. Allow gel to polymerize for 1 h.
3. Assemble gel apparatus and add the running buffer (0.5× TBE).
4. Clean wells with running buffer and make sure there are no leaks.
5. Load 50–70 µg of total RNA per lane, adjust the volume to 10 µL with DEPC-H₂O.
6. Add 10 µL of 2× RNA loading buffer to your RNA sample.
7. Heat RNA at 65°C for 10 min, chill on ice for 1 min.
8. Load samples and run at 60–80 V until the bromophenol blue reaches the bottom of the gel (~16 h).
9. After electrophoresis, stain the gel in 0.5× TBE containing 0.5 µg/mL of ethidium bromide for 30 min. This will allow you to visualize tRNAs and 5S RNA to check for RNA integrity.
10. Rinse gel in 0.5× TBE to remove excess of ethidium bromide.
11. Cut the Hybond N⁺ nylon membrane to a size slightly larger than the gel.
12. Soak the membrane in transfer buffer (0.5× TBE) for 1 min.
13. Soak four pieces of Whatman paper (GB002 grade) in 0.5× TBE.
14. Set up transfer in the Trans-blot Semi-Dry Electrophoretic transfer cell as such from bottom (anode) to top (cathode): two Whatman papers (GB002 grade), Hybond N⁺ nylon membrane, gel, two Whatman papers (GB002 grade), and cathode plate. Make sure to roll out any bubbles.
15. Transfer the RNA at 400 mA for 1 h. The voltage will start out low but will increase by the end of the transfer.

16. Wash blot in 0.5× TBE to remove any traces of the gel.
17. Place wet membrane on a wet sheet of filter paper and UV cross-link at optimal setting. Store membrane dry until use.
18. For prehybridization, use 50 mL of hybridization buffer. Prehybridization should be carried out for at least 2 h (see Note 4).
19. Oligonucleotide labeling reaction:
 - 1 μ L of oligonucleotide (10 pmol/ μ L).
 - 11 μ L of H₂O.
 - Heat for 5 min at 75°C, cool 3 min on ice.
 - Add 2 μ L of Reaction Buffer A (supplied Fermentas).
 - 5 μ L of [γ^{32} P]-ATP (10 μ Ci/ μ L).
 - 1 μ L of T4 polynucleotide kinase (10 U/ μ L, Fermentas).
 - Mix well with pipette and incubate 60 min at 37°C.
 - Stop the labeling reaction by heat inactivation of the enzyme for 10 min at 68°C.
20. Purify the labeled oligonucleotide using the Nucleotide Removal Kit (Qiagen) and elute the oligonucleotide from the column by eluting twice with 150 μ L of EB buffer, combine eluates.
21. Before adding the labeled oligonucleotide to the hybridization buffer, heat for 5 min at 75°C. Before hybridization, exchange the prehybridization buffer and hybridize in 20 mL (see Note 5) of fresh hybridization buffer for 12–18 h.
22. Wash the membrane: Washing is crucial, and there are no general rules (washing steps depend strongly on T_m of the hybridization probe, expression level of the amiRNA, etc.). Washes should be performed at a temperature 10°C below the calculated T_m of the oligonucleotide probe.
23. Discard hybridization solution. Wash briefly with 2× SSC/0.2% SDS. Wash 10 min with 2× SSC/0.2% SDS (check signals on the membrane with a Geiger tube; if necessary, continue washing). Wash 10 min with 2× SSC/0.2% SDS (check signals on the membrane with a Geiger tube; if necessary, continue washing). Wash 5–10 min with 1× SSC/0.2% SDS (check signals on the membrane with a Geiger tube; if necessary continue washing and increase washing temperature moderately).
24. Wrap membrane with saran wrap and expose to a Phosphorimager to detect hybridization signals (Fig. 2).
25. If membranes should be used for additional hybridizations (e.g., control hybridization for normalization), the membranes have to be stripped to remove bound radioactively labeled probes. To allow stripping of the membrane, make sure that the membrane does not dry during exposure; drying of the

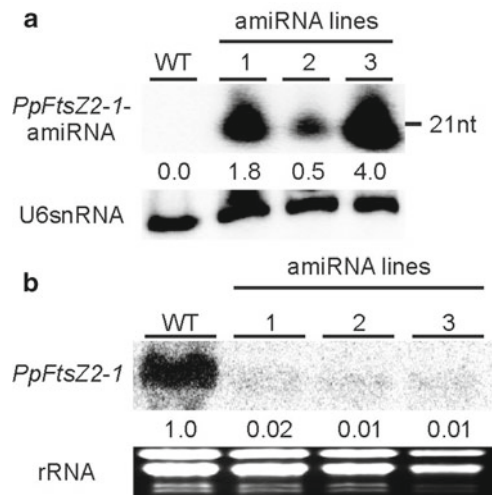


Fig. 2. Analysis of *Physcomitrella patens* lines expressing *PpFtsZ2-1*-amiRNA. (a) Expression analysis of *PpFtsZ2-1*-amiRNA expression levels in *P. patens* lines (1–3) harboring the *PpFtsZ2-1*-amiRNA expression construct and in *P. patens* wild type (control). Fifty microgram of RNA from each line was blotted and hybridized with a *PpFtsZ2-1*-amiRNA antisense probe. Hybridization with an antisense probe for U6 snRNA served as control. *PpFtsZ2-1*-amiRNA expression levels were normalized to the U6 snRNA control. Numbers indicate relative *PpFtsZ2-1*-amiRNA expression levels. (b) RNA gel blots (20 μ g each) from *P. patens* WT and *PpFtsZ2-1*-amiRNA overexpression lines (1–3) hybridized with a *PpFtsZ2-1* probe. The ethidium bromide-stained gel below indicates equal loading. The hybridization signals were normalized to the rRNA bands, and the *PpFtsZ2-1* expression level in WT was set to one. Numbers indicate the relative *PpFtsZ2-1* mRNA levels (modified from ref. 33).

membrane precludes subsequent stripping. Prepare 0.1% SDS and heat the solution to 95°C. Wash the membrane two times for 15 min at 90°C. Expose the membrane for at least 24 h to control complete removal of the probe. Stripped membranes can be frozen at –20°C wrapped with saran wrap and can be reused several times.

26. In order to compare amiRNA expression levels in different transgenic lines, it is strongly recommended to perform an additional hybridization using an oligonucleotide detecting a constitutively expressed RNA. We routinely use an antisense oligonucleotide detecting U6 snRNA that serves as internal standard for sRNA analysis. Hybridization with this oligonucleotide (5'-GGGGCCATGCTAATCTTCTCTG-3') allows to control equal loading of the RNA samples and subsequent normalization of amiRNA expression levels (Fig. 2). Labeling and hybridization are carried out, as described above. Quantification of amiRNA and U6 snRNA hybridization signals can be performed using common quantification software (e.g., Quantity One, Bio-Rad).

3.7. Expression Analysis of amiRNA Target Genes by RNA Gel Blots

The expression of an amiRNA should cause amiRNA-mediated cleavage of the intended target transcript and reduced steady-state target RNA levels. The effect of this posttranscriptional silencing and the downregulation of the target RNA have to be confirmed by RNA gel blots or quantitative real-time PCR (qRT-PCR, see Subheading 3.8):

1. Clean the gel chamber and combs properly to remove RNAsases. Leave the gel chamber filled with a detergent solution for 1 h and rinse thoroughly with water afterward.
2. Prepare 150 mL of a 1% denaturing gel for a Midi-Gel (see Note 6) in the fume hood (formaldehyde is toxic and has to be added in the fume hood): Mix 1.5 g agarose with 108 mL of distilled H₂O, boil until agarose is completely dissolved, cool the solution down, then add 15 mL of 10× MOPS buffer, 27 mL of 37% (*w/v*) formaldehyde, mix and pour the gel solution into gel tray, and insert comb.
3. Mix RNA sample (up to 30 µg) with 1 volume of RNA denaturation buffer, incubate 10 min at 65°C, cool sample immediately on ice, add 0.1 volume of 10× RNA loading buffer, mix and immediately load onto the gel; until loading, keep the samples on ice. If you include RNA markers, these have to be treated in exactly the same way.
4. Electrophoresis is carried out in 1× MOPS buffer (under fume hood).
5. To achieve a good separation of the RNA, run the gel at a constant voltage of 100 V.
6. Let the gel run until the bromophenol blue reaches 2/3 of the gel length.
7. Before blotting, take a photo of the gel for documentation (e.g., confirmation of equal loading of the gel).
8. Cut the Hybond N⁺ nylon membrane to a size slightly larger than the gel and soak the membrane in distilled water for 15 min.
9. Set up RNA transfer in a Turboblotter system as such from bottom to top: 20 Whatman papers (dry GB004 grade), four Whatman papers (dry GB002 grade), one Whatman paper (wet GB002 grade), wet Hybond N⁺ nylon membrane, RNA gel, three Whatman papers (wet GB002 grade), and cover plate. Make sure to roll out any bubbles.
10. Transfer at room temperature for 4 h (additional transfer time may be required for thicker gels).
11. Place wet membrane on a wet sheet of filter paper and UV cross-link at optimal setting. Store membrane dry until use.
12. To prepare 100 mL of hybridization buffer, mix 34.2 mL of 1 M Na₂HPO₄, 15.8 mL of 1 M NaH₂PO₄, 200 µL of 0.5 M

EDTA, pH 8.0, 13.8 mL of H₂O and 35 mL of 20% SDS, and heat the solution to 67°C.

13. Boil 1 mL of salmon sperm DNA (10 mg/mL) for 10 min, cool on ice, and add to the prewarmed hybridization buffer.
14. For prehybridization, use 50 mL of the hybridization buffer. Prehybridization should be carried out for at least 2 h at 67°C.
15. The labeling of the hybridization probe (cDNA or genomic fragments of the target to be analyzed) is performed using the Rediprime II Random Prime Labelling System (Amersham Biosciences) according to the manufacturer's instructions.
16. Before hybridization, exchange the buffer. The volume of buffer has to be kept as small as possible (see Note 5). Add the denatured radioactively labeled probe to the buffer and hybridize for 12–18 h at 67°C.
17. Prepare the wash solutions and preheat solutions to 67°C.
18. Add the wash solution (1× SSC, 0.1% SDS) and rinse the membrane briefly, discard into radioactive waste. Wash with 1× SSC, 0.1% SDS for 10 min at 67°C. Wash three times with 0.5× SSC, 0.1% SDS for 10 min at 67°C.
19. Wrap the membrane with saran wrap and expose to a Phosphorimager to detect hybridization signals (Fig. 2).
20. If membranes should be used for additional hybridizations (e.g., control hybridization for normalization), they have to be stripped to remove bound radioactively labeled probes. To allow stripping of the membrane, make sure that the membrane does not dry during exposure; drying of the membrane precludes subsequent stripping. Prepare 0.1% SDS and heat the solution to 95°C. Wash the membrane two times for 15 min at 90°C. Expose the membrane for at least 24 h to control complete removal of the probe. Stripped membranes can be frozen at –20°C wrapped with saran wrap and can be reused several times. For control hybridizations to monitor equal loading of RNA samples and subsequent normalization of RNA expression levels, we routinely use the constitutively expressed gene *PpEF1α* encoding, a translation elongation factor. Amplify a *PpEF1α* cDNA fragment from *P. patens* using the primers 5'-AGCGTGGTATCACAATTGAC-3' and 5'-GATCGCTCGATCATGTTATC-3'. Use this fragment for radioactive labeling and hybridization of RNA blots, as described above.

3.8. Expression Analysis of amiRNA Target Genes by Quantitative Real-Time PCR

1. Treat 1 µg of total RNA with 1 U DNase I (1 U/µL, Fermentas) and reverse-transcribe into first-strand cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's instructions. Include a minus RT control for each RNA probe within your analysis to check for DNA contamination.

2. Design the gene-specific primers according to the following rules (primer design may be performed using special software, e.g., Primer Express, Applied Biosystems): Avoid sequences with consecutive identical nucleotides (i.e., GGGG or CCCC), the amplified product should have a length from ~50 bp to max ~800 bp (the shorter the better), $T_m \geq 60^\circ\text{C}$, no more than two G or C in the last 5 nt at the 3' end. The PCR primers should flank the amiRNA recognition motif. The 5' and 3' cleavage products that are generated by miRNA-directed RNA cleavage may be stable and may be amplified by primers that do not flank the miRNA recognition motif. If primers are designed to span the miRNA binding site, only intact non-cleaved mRNAs are detected.
3. Design the experiment to include: H₂O control, three independent biological replicates, and three technical replicates for each sample.
4. Use *PpEF1a* as constitutively expressed genes for normalization.
5. Adjust the final cDNA concentration to 10 ng/ μL and use 50 ng for each PCR reaction. Prepare 150 ng (15 μL) for each sample in a 1.5-mL Eppendorf tube which resembles three technical replicates.
6. For each sample, prepare 60 μL of Light Cycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions and add to 15 μL of cDNA mentioned in step 5. Mix and spin down briefly.
7. Divide the 75 μL of CR mixture equally into three wells (23 μL /well) of 96-well real-time PCR plate. Seal the plate, centrifuge briefly, and run PCR in real-time PCR cycler (e.g., Roche 480 Light Cycler).

3.9. Detection of RNA Cleavage Products by RNA-Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

Besides the analysis of amiRNA target expression, the amiRNA-directed cleavage of the target RNA should be confirmed by RLM 5' RACE. To detect amiRNA-mediated cleavage products, we apply a modified protocol of the GeneRacer Kit (Invitrogen). This protocol was originally developed to amplify 5' ends from full-length RNAs (54). In the original protocol, the mRNA is first dephosphorylated to remove 5' phosphate residues which are present at the 5' end of RNA degradation products. In a subsequent step, the cap is removed and replaced with an RNA adaptor oligonucleotide (GeneRacer RNA oligonucleotide). During reverse transcription, this RNA adaptor sequence is incorporated into the cDNA. 5' RACE PCR is then performed using the homologous GeneRacer 5' primer that is specific to the RNA oligonucleotide sequence and a gene-specific primer. The result is amplified DNA that contains the full-length 5' cDNA sequence. To detect RNA cleavage products that result from amiRNA-directed RNA cleavage, the protocol is modified (55). The 3' RNA cleavage products

contain phosphorylated 5' ends that are directly accessible for the RNA adaptor ligation. Furthermore, the detection of full-length capped mRNAs is not intended. Thus, in the modified protocol, dephosphorylation and decapping of the RNA are abolished, and the RNA adaptor is directly ligated to the RNA samples. During cDNA synthesis, the RNA adaptor sequence is incorporated to the cDNA, and subsequent 5' RACE PCR with an adaptor primer together with a gene-specific primer allows the amplification of the cleavage products:

1. Isolate poly(A)-mRNA from total RNA using Oligotex mRNA Mini Kit (Qiagen) as recommended by the manufacturer.
2. Combine ~150 ng of mRNA and 250 ng of GeneRacer RNA oligonucleotide and incubate at 65°C for 5 min. Set up the ligation reaction by adding:
 - 1 μ L ATP (10 mM).
 - 2 μ L BSA (0.1 mg/mL).
 - 2 μ L of 10 $\times$ RNA-ligase buffer.
 - 1 μ L RNaseOUT (40 U/ μ L).
 - 1 μ L T4 RNA ligase (5 U/ μ L).Add water up to 20 μ L and incubate at 37°C for 1 h.
3. Increase the volume to 100 μ L by adding RNase-free water and purify the ligated RNA by phenol/chloroform/isoamyl alcohol (25:24:1) extraction. For this purpose, add an equal volume of phenol/chloroform/isoamyl alcohol to the RNA, vortex for 30 s, and centrifuge at 16,000 $\times g$ for 5 min. Transfer the uppermost aqueous phase containing the RNA into a fresh Eppendorf cap. Precipitate the RNA by adding 0.1 volume of 3 M sodium acetate, pH 5.2, 2.5 volume of ethanol and GlycoBlue (Applied Biosystems) to reach a final concentration 100 μ g/mL. GlycoBlue acts as a carrier during RNA precipitation and facilitates visualization of the pellet. Wash the RNA pellet with 70% ethanol and dissolve in 7 μ L of RNase-free water.
4. Start cDNA synthesis using the complete RNA obtained from step 3 with Superscript III reverse transcriptase (Invitrogen). Set up the reaction as follows:
 - 7 μ L of adaptor-ligated RNA.
 - 1 μ L dNTP mix (10 mM each).
 - 1 μ L of Oligo(dT)₁₈ primer (50 pmol/ μ L).Incubate at 65°C for 5 min, place on ice for 2 min, and continue by adding:
 - 4 μ L of 5 $\times$ RT buffer.
 - 4 μ L MgCl₂ (25 mM).
 - 2 μ L DTT (0.1 M).

- 1 μL RNaseOUT (40 U/ μL).
Incubate at 42°C for 2 min, then add.
0.5 μL Superscript III RT (200 U/ μL).
Incubate at 42°C for 50 min. Stop the reaction at 70°C for 15 min, chill on ice, and spin down briefly. Add 1 μL of RNase H (2 U/ μL) and incubate for 20 min at 37°C.
5. Start 5' RACE PCR with the GeneRacer primer and a gene-specific primer (see Note 7). Set up the PCR reaction as follows:
- 1 μL of GeneRacer 5' Primer (10 μM).
1 μL of reverse gene-specific primer (10 μM).
1 μL of 5' RACE cDNA.
3 μL of 10 $\times$ Pfx Buffer.
0.5 μL dNTP mix (10 mM each).
0.25 μL Platinum Pfx DNA polymerase (2.5 U/ μL).
0.5 μL MgSO_4 (50 mM).
Add H_2O to 30 μL .
Perform the PCR using the following conditions:
- Step 1: 94°C, 2 min.
Step 2: 94°C, 30 s
Step 3: 72°C, 1 min/kb (repeat steps 2–3 five times).
Step 4: 94°C, 30 s.
Step 5: 70°C, 1 min/kb (repeat steps 4–5 five times).
Step 6: 94°C, 30 s.
Step 7: 60–68°C, 30 s.
Step 8: 68–72°C, 1 min/kb (repeat steps 6–8 20–25 times).
Step 9: 68–72°C, 10 min.
6. Analyze the PCR products by DNA gel electrophoresis. If you observe DNA smear or multiple bands from the 5' RACE PCR, a subsequent nested PCR is recommended. Amplify nested RACE PCR products using a nested gene-specific primer and the GeneRacer 5' nested Primer (see Note 8). Set up the nested PCR as follows:
- 2.5 μL of 10 $\times$ Pfx Buffer.
0.5 μL dNTPs (10 mM each).
0.5 μL MgSO_4 (50 mM).
1 μL of nested GeneRacer 5' Primer (10 μM).
1 μL of reverse nested gene-specific primer (10 μM).
1 μL from the initial 5' RACE PCR (template).
0.25 μL Platinum Pfx DNA polymerase (2.5 U/ μL).

18.25 μL of H_2O .

PCR conditions:

Step 1: 94°C, 2 min.

Step 2: 94°C, 30 s.

Step 3: 65°C, 30 s.

Step 4: 68°C, 1 min/kb (repeat steps 2–4 15–25 times).

Step 5: 68°C, 10 min.

7. Analyze the PCR products by DNA gel electrophoresis. Elute fragments that correspond to the expected size of the cleavage product, clone, and sequence (see Note 9).

4. Notes

1. Design amiRNAs lacking perfect sequence complementarity at the 3' end, as this reduces transitivity.
2. In most of the cases, the amiRNA was expressed from endogenous miRNA precursors. The *A. thaliana* miR319a precursor can be used routinely for the expression of amiRNAs in *P. patens*.
3. Be careful working with RNA, RNA is easily degraded by RNases which can be carried over to your RNA samples. Use autoclaved pipette tips and baked glassware. Wear gloves when you work with RNA. Prepare all required solutions with DEPC-treated H_2O .
4. The prehybridization and hybridization temperature depend on the sequence of the amiRNA antisense oligonucleotide. Calculate the T_m (annealing temperature) of the oligonucleotide using the following formula (this formula is valid for small oligonucleotides with a size of 17–24 nt): $T_m = (A+T) \times 2 + (C+G) \times 4$. The hybridization temperature should be 10°C below the calculated T_m of the oligonucleotide.
5. Hybridization conditions may differ substantially based on the intention of the designed experiment. The most important criteria are the stringency conditions. High hybridization and wash temperatures coupled with low salt concentrations are high-stringency hybridization conditions. Low temperature and high salt concentrations favor unspecific hybridizations and represent low-stringency hybridization conditions. Keep the volume of hybridization buffer as small as possible to achieve high probe concentration during the hybridization. The membrane has to be covered completely.
6. To achieve a good separation of RNA samples, we recommend using a Midi-Gel rather than using Mini-Gels. Furthermore, if

high amounts of RNA (>10 µg of total RNA) are loaded onto the gel, it is better to use a 10- or 12-well comb. When using a 20-well comb, the amount of RNA per well might be too much and may result in bad signals after the hybridization.

7. Gene-specific primers are designed with 50–70% GC content and having high annealing temperature (>72°C).
8. Nested gene-specific primers, located upstream of the initial gene-specific RACE PCR primer, should have a 50–70% GC content and an annealing temperature above 68°C.
9. It is recommended to sequence several independent clones to map the amiRNA cleavage site.

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High Frequency of Single-Copy T-DNA Transformants Produced After Floral Dip in *CRE*-Expressing *Arabidopsis* Plants

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Abstract

Transgenic plants that harbor a single copy of the introduced transgene are preferable to those with multiple transgene copies because multiple T-DNA copies correlate with expression variability and susceptibility to silencing. Especially after the commonly used floral-dip *Agrobacterium*-mediated transformation method, the frequency of single-copy transformants is low. The CRE/*loxP* recombinase-based strategy to resolve complex T-DNA loci has proven to be successful to efficiently obtain single-copy T-DNA transformants by directly transforming *loxP*-containing T-DNA vectors in *CRE*-expressing *Arabidopsis thaliana* plants. This chapter describes in detail how to transform three available *loxP*-containing T-DNA vectors into *CRE*-producing *Arabidopsis* C24 plants and subsequently how to analyze the transgenic plants for the T-DNA locus structure.

Key words: CRE/*loxP* recombinase, Single-copy T-DNA, *Arabidopsis*, Floral-dip transformation, T-DNA inversion

1. Introduction

The most commonly used technology to introduce genes into plants is *Agrobacterium tumefaciens*-mediated transformation. The transferred DNA segment (T-DNA) is delineated by two nonidentical 25-bp direct border repeats and is integrated into the plant genome via illegitimate recombination (1–3). However, after *Agrobacterium*-mediated floral-dip (4) transformation, multicopy T-DNA loci are frequently found, as a result of replication and/or extrachromosomal ligation of different T-DNAs before integration (5). To obtain transgenic plants with high and stable overexpression

of the gene of interest under the control of the 35S promoter, it is important to generate transformants with single-copy T-DNAs because in this case, the 35S-driven expression is generally uniform and stable, irrespective of the integration position (6, 7). The conventional method to identify transformants with an elite single-copy insert is to screen a large pool of transformants by Southern blot analysis or T-DNA fingerprinting (8), but both methods are time consuming, and finally, only about 10% of the transformants are single-copy T-DNA plants.

The well-characterized CRE/*loxP* recombination system is one of the most powerful research tools in genetics and has also been widely used in plant biotechnology for targeted insertion and precise deletion of DNA from transgenic plant DNA (see refs. 9–11 and therein). Basically, the CRE/*loxP* system contains two components: the CRE recombinase and its 34-bp *loxP* target DNA sequences (12). When the two *loxP* sites are in direct orientation, the CRE recombinase catalyzes excision of the intervening DNA between them. In contrast, when the two *loxP* sites are present in opposite orientation, the CRE protein inverts the DNA fragment between the *loxP* sites (12).

Previously, De Buck and collaborators had been investigating whether complex T-DNA loci could be efficiently resolved into single copies by CRE/*loxP*-mediated recombination in *Arabidopsis*. Therefore, a SB-*loxP* T-DNA was designed that contained two inversely oriented *loxP* sites located inside and immediately adjacent to the T-DNA border ends. Seven plants with a complex SB-*loxP* locus were crossed with a CRE-expressing plant (13). In this resolution-based strategy, thanks to the presence of the CRE recombinase, recombination between the outermost *loxP* sequences in direct orientation resolves the integrated T-DNAs into a single copy, regardless of the number of T-DNAs integrated into one locus. Reduction to a single SB-*loxP* T-DNA copy had been demonstrated in three out of seven crossings together with an at least tenfold-enhanced reporter gene activity in two transformants (13).

As an alternative to this approach, De Paepe and coworkers investigated whether single-copy T-DNA transformants could be obtained by floral-dip transformation directly into CRE-expressing plants by using a Ksc2 T-DNA containing two oppositely oriented *loxP* sites located next to the left and right T-DNA borders (14). Fifty-five percent of the primary CRE-expressing transformants harbored a single copy of the introduced T-DNA while only 15% of wild-type transformants contained a single copy. In this case, it could also be observed that 45% of the obtained single-copy transformants constitutively inverted the DNA segment between the two *loxP* sites because of constitutive CRE activity.

To avoid this internal inversion of the T-DNA segment between the *loxP* sites in CRE-expressing plants and possibly associated locus instability, we tested whether transformation of a Ksc1

T-DNA harboring only one *loxP* site at the left border (LB) and a HscI T-DNA with one *loxP* site at the right border (RB) would also allow resolution to single-copy T-DNA transformants (14). Here, 60 and 78% of the transformants in the CRE background were single-copy transformants when the KscI T-DNA vector and the HscI T-DNA vectors were used, respectively. In contrast, in the wild-type background, the single-copy T-DNA frequency was only 11% for both T-DNA vectors. Furthermore, this resolution strategy resulted in enhanced and uniform transgene expression in the progenies of most of the self-fertilized primary transformants. Therefore, the use of these T-DNA vectors for transformation in CRE-expressing plants should help to increase the frequency with which high-quality transformants in *Arabidopsis* can be obtained after floral-dip transformation. Also, it opens the possibility to generate elite events in plants from which single-copy T-DNA transformants are relatively difficult to obtain.

In this chapter, we describe the method for generating more readily single-copy T-DNA transformants by floral-dip transforming *loxP*-containing T-DNA vectors into CRE-expressing *Arabidopsis* plants. Plant transformation, selection of transformants, determination of the T-DNA integration pattern, and analysis of the T-DNA inversion between the *loxP* sites when the pKsc2 transformation vector is used are described in detail.

2. Materials

2.1. Plant Transformation

All buffers and solutions are made as aqueous solutions unless specified otherwise.

1. Deionized water.
2. Luria–Bertani (LB) medium: 10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of sodium chloride. Sterilize by autoclaving and store at room temperature.
3. 50-mL capped centrifuge tubes (such as Corning, Lowell, MA).
4. Solution of 0.5% (*v/v*) Silwet L-77 (Lehle Seeds, Round Rock, TX) and 10% (*w/v*) sucrose.
5. Shaker incubator (28°C).
6. UV/Visible spectrophotometer (for instance, model DU 530; Beckman Coulter, Brea, CA).
7. Seed-harvesting Aracon bases and sheets (Lehle Seeds).
8. Saran wrap (Dow Chemical, Midland, MI).
9. Regular potting soil.

2.2. Selection of Transgenic T-DNA Plants

1. K1 growth medium: 2.15 g/L half-strength Murashige and Skoog salts (Gibco-BRL, Gaithersburg, MD) and 0.5 g/L 2-(*N*-morpholino) ethanesulfonic acid (MES) (Duchefa Biochemie, Haarlem, The Netherlands) supplemented with 10 g/L sucrose. Bring this solution to pH 5.7 with KOH and complete with 8 g/L agar. Sterilize by autoclaving. After cooling to 60°C, add the selective agents and fill ten to twelve 15-cm Petri dishes with the sterilized K1 medium. Selective plates can be stored up to 6 months at room temperature.
2. Selective conditions are 50 mg/L kanamycin (Duchefa) and 20 mg/L hygromycin for the Ksc2, Ksc1, and Hsc1 T-DNA transformation vectors (Calbiochem, La Jolla, CA). Store in single-use aliquots at -20°C (see Note 1).
3. Countersselective agent to prevent fungal growth is Nystatin (Duchefa) at a concentration of 50 mg/L. Dissolve in dimethylsulfoxide (DMSO) and store in single-use aliquots at -20°C.
4. Selective agent against *Agrobacterium*: 300 mg/L of carbenicillin (Duchefa). Carbenicillin powder (store at -20°C) is added as such to the sterilized K1 medium when cooled to 60°C.
5. Miracloth filter paper (Calbiochem).

2.3. Southern Blot Analysis

1. DNA loading buffer: 15% (*w/v*) Ficoll (type 400), 0.25% (*w/v*) bromophenol blue, and 0.25% xylene cyanol (*w/v*).
2. SmartLadder (Eurogentec, Luik, Belgium).
3. 10× TAE: 48.4 g Tris base, 11.4 mL glacial acetic acid, and 20 mL 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 8/L. Sterilize by autoclaving and store at room temperature.
4. 1% (*w/v*) Agarose gel in 1× TAE.
5. Running buffer: 1× TAE, prepared from 10× TAE.
6. Rotary shaker (for instance, model Orbital Shaker; Bellco Biotechnology, Vineland, NJ).
7. 0.25 M HCl.
8. Denaturation solution (pH 14): 0.5 M NaOH and 1.5 M NaCl. Store at room temperature.
9. Neutralization solution (pH 7.5): 1 M Tris-HCl and 1.5 M NaCl. Store at room temperature.
10. 20× SSC (pH 7): 3 M NaCl and 0.3 M sodium citrate. Store at room temperature.

2.4. Membrane Hybridization

1. PCR DIG probe synthesis mix (Roche, Mannheim, Germany).
2. Hybond-N⁺ nylon transfer membrane (GE Healthcare, Little Chalfont, UK).
3. Dig Easy Hyb buffer (Roche).
4. Hybridization incubator and tubes (for instance, model HB-1D; Techne, Burlington, NJ).

5. Washing solution 1: 2× SSC, 0.1% (*w/v*) sodium dodecyl sulfate (SDS).
6. Washing solution 2: 1× SSC, 0.1 (*w/v*) SDS.
7. Buffer 1: 100 mM maleic acid and 150 mM NaCl. Adjust to pH 7.5 with NaOH. Sterilize by autoclaving and store at room temperature.
8. Washing solution 3: buffer 1 containing 0.3% polyoxyethylene sorbitan (Tween-20). Sterilize by autoclaving and store at room temperature.
9. Blocking buffer: buffer 1 containing 1% blocking powder (Roche). Sterilize by autoclaving and store at 4°C.
10. Anti-digoxigenin (DIG)-AP Fab fragments (Roche).
11. B3 buffer: 100 mM Tris-HCl (pH 9.5) and 100 mM NaCl. Make a 10× stock of both components, sterilize by autoclaving, and store at room temperature. Mix just before use.
12. CDP-star (Roche Applied Science).

2.5. PCR Analysis

1. Taq DNA polymerase 5 U/μL (for instance, Invitrogen, Carlsbad, CA).
2. 10× PCR buffer: 200 mM Tris-HCl, pH 8.4, and 500 mM KCl (for instance, Invitrogen).
3. 50 mM MgCl₂ (for instance, Invitrogen).
4. 10 mM dNTP mix (for instance, Promega, Madison, WI).
5. DMSO.
6. 0.2 and 1.5-mL microfuge tubes (Eppendorf, Hamburg, Germany).
7. Thermal cycler (for instance, iCycler; Bio-Rad, Hercules, CA).
8. Running buffer: 1× TAE, prepared from 10× TAE.

3. Methods

3.1. Generation of T-DNA Transformants in a CRE-Expressing Background

Homozygous single-copy *CRE*-expressing plants (Cre1 line) (15) are used for floral-dip experiments. The Cre1 line was obtained after root explant transformation of *Arabidopsis thaliana* (ecotype C24) (16) (see Note 2) with the CRE T-DNA (see Fig. 1) (17) and selection on phosphinothricin (10 mg/L). After segregation and Southern blot analyses, a single-copy Cre line, Cre1, was retained, and a homozygous seed stock was generated.

The Cre1 plants are then transformed by means of the *Agrobacterium*-mediated floral-dip method (4) with an *Agrobacterium* containing Ksc2-, Ksc1-, or Hsc1-derived T-DNAs (see Fig. 1), or modified with gene(s) of interest (see Note 3).

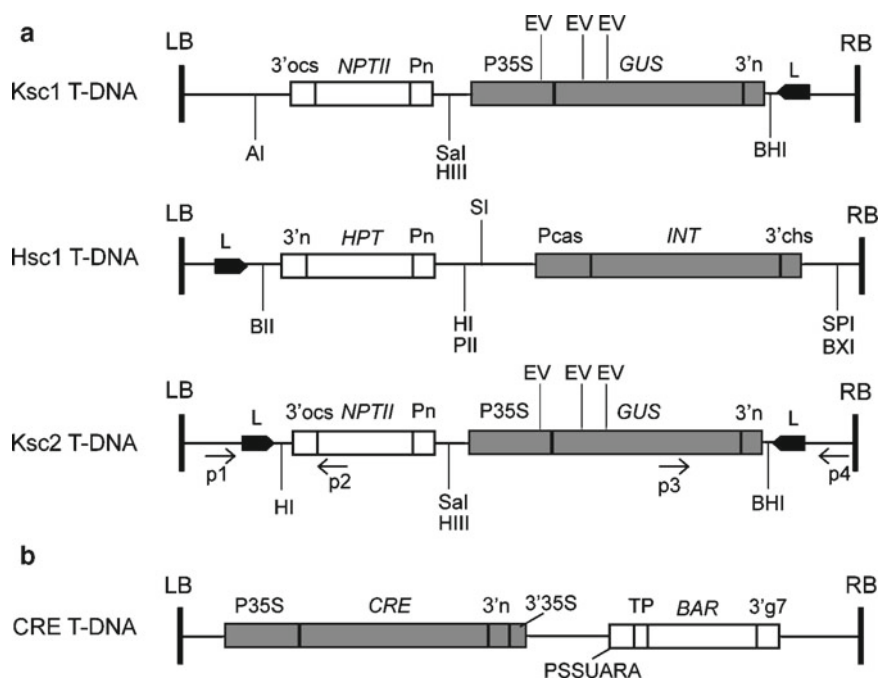


Fig. 1. Schematic representation (not to scale) of T-DNAs. (a) The Ksc2, Ksc1, and Hsc1 T-DNAs with indication of their unique restriction sites usable for Southern blot analysis and for cloning a gene of interest. The *loxP* sequences are represented by *thick black arrows* that show their orientation. The primers p1, p2, p3, and p4 are marked by *arrows*. (b) The CRE T-DNA. As the *CRE* recombinase in the Cre1 plant is under the transcriptional control of the 35S promoter, inversion of the sequence between the *loxP* sequences is possible when the pKsc2 vector is used. In contrast, the Ksc1 or Hsc1 vectors will not yield inversion of an internal T-DNA segment because only one *loxP* site is present. 3'ocs 3' end of the octopine synthase gene, *NPTII* neomycin phosphotransferase II gene, *Pn* promoter of the nopaline synthase gene, *P35S* CaMV 35S promoter, *GUS* β -glucuronidase-coding sequence, 3'n 3' end of nopaline synthase gene, *HPT* hygromycin phosphotransferase gene, *Pcas* cassava vein mosaic virus promoter (20), 3'chs 3' end of the chalcone synthase gene, *INT* integrase gene of bacteriophage ϕ C31 (21), *L loxP* sequence recognition site of the *CRE/loxP* recombination system, *CRE CRE* recombinase gene; 3'35S, 35S terminator, *PSSUARA* the small subunit promoter of *Arabidopsis*, *TP* transit peptide, *BAR* phosphinothricin transferase gene, 3'g7 3' terminator of gene 7, *LB* left border, *RB*, right border; unique restriction sites: *Al*, *Apal*; *BHI*, *BamHI*; *BII*, *BglII*; *BXI*, *BstXI*; *EV*, *EcoRV*; *HI*, *HpaI*; *HIII*, *HindIII*; *PII*, *PvuII*; *Sal*, *SalI*; *SPI*, *SpeI*; *SI*, *SphI*.

Preferably, the Ksc1 or Hsc1 vector might be used as backbone for designing and constructing T-DNA because only one *loxP* site is present. Use of the Ksc2 transformation vector, which contains two *loxP* sites in opposite orientation, might invert the T-DNA sequence between the two *loxP* sites. T-DNA constructs are subsequently electroporated into the *Agrobacterium* strain C58C1Rif (pMP90) (18).

3.1.1. Plant Transformation

1. Bring 1 ml of LB medium without antibiotic in a 50-mL tube; inoculate with the *Agrobacterium* strain harboring the *loxP*-containing T-DNA vector taken from a selection plate kept at 4°C (see Note 4).
2. Grow for 8–9 h at 28°C in a rotary shaker with agitation of 230 rpm.

3. Add 10 mL of LB medium without antibiotic and incubate overnight at 28°C in a rotary shaker with agitation of 230 rpm.
4. Determine the optical density of the culture spectrophotometrically at 600 nm. As a control sample, use 1 mL of LB. The OD value must be in between 1.5 and 2.0; when the value is lower, further incubate the culture at 28°C and measure again after each 30 min.
5. Add 40 mL of H₂O containing sucrose (10%, 4 g) and Silwet (0.05%, 20 µL) to the 10-mL culture. Use the mix immediately for the floral dip.
6. Dip the inflorescences of the Cre1 plants of about 4 weeks old (T0) into the suspension for 5–10 s (see Note 5). Typically, ten plants per construct are dipped to obtain a sufficient number of independent transformants (see Note 6).
7. Cover the plants with a transparent plastic bag or paper and return the plants to normal growing conditions (21°C, 16-h light/8-h dark cycle) (see Note 7).
8. After 24 h, remove the plastic bag or the paper.
9. Five weeks after floral dip, stop watering the transformed plants.
10. When the plants are dry, harvest the T1 seed stock and store in plastic tubes at 4°C.

3.1.2. Selection of Transgenic T-DNA Plants

To recover the primary transformants (T1) from the T1 seed stock of the T0-dipped plants, an antibiotic is added to the growth medium so that only transgenic plants harboring the selective marker can grow (see Note 8). Also, carbenicillin and nystatin which do not affect plant cells are utilized to arrest the growth of the transforming *Agrobacterium* still present after the floral dip and to avoid fungal infection, respectively. In the following examples, kanamycin (Ksc2 and Ksc1 vectors) and hygromycin (Hsc1 vector) were used as antibiotics to select the T-DNA-containing transgenic plants (see Fig. 1).

1. Pack 25 mg (~1,000 seeds) of the T1 seed stocks obtained from every dipped plant in Miracloth filter paper. Surface sterilize seeds and sow on selective K1 medium.
2. Keep sown plates 1–4 days at 4°C (see Note 9).
3. Incubate plates in a growth chamber at 21°C on a 16-h light/8-h dark cycle.
4. Transformed plants are easy to identify after 15–20 days of growth on the selective medium as green seedlings with third and fourth leaves.
5. After 3–4 weeks, transfer T1 plants that are large enough with two pairs of green leaves and normally expanded roots to soil.

3.2. Determination of the T-DNA Integration Pattern in the Obtained Transformants and Selection of Single-Copy Transformants

Based on our previous results, we obtained 55, 60, and 78% single-copy transformants after transformation with the Ksc2, Ksc1, and Hsc1 construct in the Cre1 plant, respectively (14). These percentages indicate that even when a small number of transformants is obtained, more than 50% is expected to be single-copy transformants.

By far, the best technique for analyzing T-DNA integration patterns is Southern blotting. After the genomic DNA is digested with a suitable restriction enzyme, border fragments can be visualized with an LB and an RB probe. As soon as the number of border fragments obtained with the RB and with the LB probes are determined, the single-copy T-DNA transformants that have only one RB/plant junction fragment and one LB/plant junction fragment can be selected.

3.2.1. Southern Blotting

1. Isolate genomic DNA from leaves of the selected T1 transformants or from several T2 segregants (see Note 10).
2. Choose the appropriate restriction enzyme for the Southern analysis (see Note 11), taking into account that the enzymes should not be prone to cytosine methylation (19) and should cut at least 1,000–2,000 bp away from the T-DNA borders (see Note 12).
3. Perform the restriction digest on 1 µg of genomic DNA from the selected transformants. Routinely, add 3 U restriction enzyme per microgram DNA. Incubate for 3 h. Add again 3 U/µg restriction enzyme and incubate for another 3 h. When the total volume of the restriction digest exceeds the volume that can be loaded on the gel, reduce the volume of the digests by evaporation in a speed-vac. Add one-tenth volume of DNA loading buffer.
4. Pour a 1% (*w/v*) agarose gel and load the digests. Include a DNA molecular weight marker (see Note 13).
5. Run the gel at 100 V until the markers in the loading dye have migrated into the gel. Afterward, continue at 25 V and let the gel run overnight.
6. After the bromophenol dye has migrated about two-thirds of the total length of the gel, place the gel in a clean glass dish.
7. Cover the gel with a 0.25 M HCl solution for 15 min while shaking slowly on a platform shaker. The color of the xylene cyanol and bromophenol blue dye will change to green and yellow, respectively.
8. Remove the HCl solution and rinse the gel three times with deionized H₂O.
9. Cover the gel with the denaturation solution for 15 min under shaking. The color of the dyes will return to their original colour. Remove and replace with new denaturation solution.

Incubate again for another 15 min. Afterward, rinse the gel three times with deionized H₂O.

10. Cover the gel with neutralization buffer for 30 min under shaking. Pour off solution, add fresh buffer, and incubate for another 15 min.
11. Blot the gel overnight to a nitrocellulose membrane by capillary transfer action with a 20× SSC solution.
12. Bake the membrane in a regular oven at 80°C for 2 h or expose to UV radiation (50 mJ for 50 s) to permanently attach the transferred DNA to the membrane (see Note 14).

3.2.2. Hybridization
of the Membrane

The method described is a protocol that is routinely applied in our laboratory; using digoxigenin (DIG)-labeled probes (see Note 15). Two different probes, an LB and RB probe, are used in succession on the same membrane. These LB and RB probes should specifically detect LB T-DNA/plant junctions and RB T-DNA/plant junction fragments, respectively. Therefore, the DNA fragment for the LB probe should be located between the LB and the closest restriction site (see Fig. 2). The same holds true for the RB probe that should localize between the RB and the restriction site closest to this border. Usually, DNA fragments of 500–1,000 bp are chosen to prepare the probes.

1. Run a regular PCR using 100 ng of DNA for amplification of the DNA fragments between specific primers flanking the segments that are selected as LB and RB probes.
2. Use 1 μL of this PCR reaction as a template for the PCR with DIG-labeled dNTPs:

1 μL non-DIG template	1 μL
10× PCR buffer	5 μL
MgCl ₂ (50 mM)	1.5 μL
PCR DIG probe synthesis mix	5 μL
Primer <i>x</i> (50 ng/μL)	5 μL
Primer <i>y</i> (50 ng/μL)	5 μL
Taq polymerase (5 U/μL)	0.6 μL
H ₂ O	26.9 μL

3. Clean up the DIG PCR reaction. We routinely use the High Pure PCR Product Purification Kit (Roche). The final volume would be 100 μL.
4. Check the probe on an agarose gel by loading 5 μL of the purified DIG probe in addition to 5 μL of the original PCR product. After EtBr staining, the DIG probe must run a little higher than the non-DIG probe due to the incorporated DIG nucleotides.

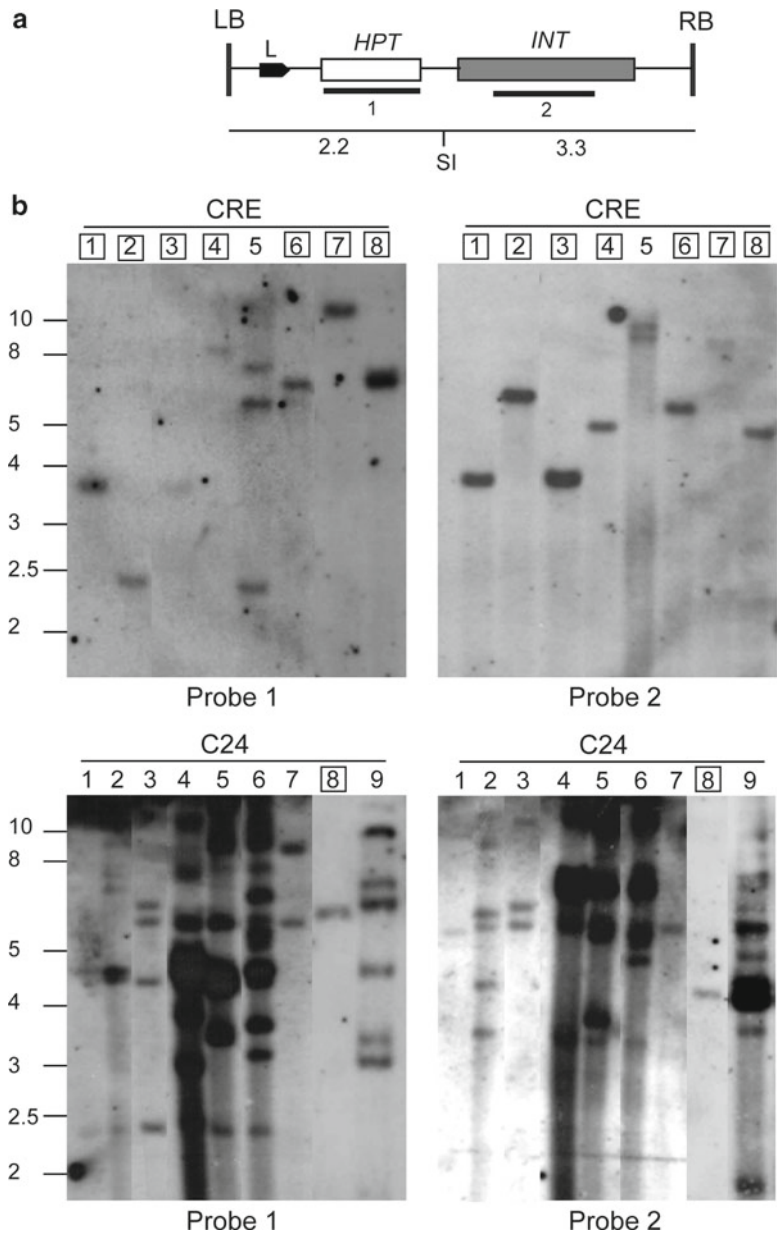


Fig. 2. DNA gel-blot analysis on *SphI*-digested T1 leaf DNA from plants transformed with the Hsc1 T-DNA in CRE and wild-type (C24) background. (a) After *SphI* (SI) digestion of plant DNA, left T-DNA/plant junctions of more than 2.2 kb are expected after hybridization with the *HPT* (probe 1), and right T-DNA/plant junctions of at least 3.3 kb are expected after hybridization with the *INT* probe (probe 2). (b) DNA gel-blot fragments detected with the LB T-DNA/plant junction probe (probe 1) (left) and with the right T-DNA end-specific *INT* probe (probe 2) (right). Top and bottom panels represent the Southern blots resulting from independent transformants obtained after transformation of the Hsc1 T-DNA in CRE-expressing plants and Hsc1 T-DNA-transformed wild-type (C24) plants, respectively. Squares indicate single-copy transformants in which only one RB/plant junction fragment and one LB/plant junction fragment are visualized. *HPT* hygromycin phosphotransferase, *INT* integrase. Adapted from ref. 13, © Blackwell Publishing Ltd.

5. Put the filter carefully in a hybridization tube and add Dig Easy Hyb buffer. Use at least 15 mL in a small tube or 40 mL in a large one (see Note 16). Try to avoid air bubbles. Prehybridize the membrane for 1 h at 42°C. In the meantime, the probes can be denatured.
6. Make several holes in the cap of the probe-containing Eppendorf tubes with a hot needle. Heat denature the probe for 5 min at 95°C in a water bath or incubator and put the probe afterward straight into ice.
7. Add the denatured probe directly in the tube with the hybridization buffer while avoiding direct contact with the membrane and leave to hybridize overnight at 42°C (see Note 17).
8. Wash the membrane twice for 5 min with preheated washing solution 1 at room temperature. Heat the oven to 65°C for the next washes.
9. Wash the membrane for 15 min with preheated washing solution 1 at 65°C, followed by 15 min incubation with washing solution 2 at 65°C.
10. Wash 5 min in washing solution 3 at room temperature, pour off, and incubate the membrane for 30 min in blocking buffer at room temperature. During these 30 min, prepare the blocking anti-DIG solution by centrifuging the anti-DIG-AP Fab fragments for 5 min and add 2 µL of the antibody to 40 mL of blocking buffer.
11. Replace the blocking buffer with the blocking anti-DIG solution in a rotary shaker for another 30 min. Pour off and wash the filter at least three times for 15 min in washing solution 3.
12. Incubate the filter for 5 min in B3 buffer.
13. Wrap the membrane in a plastic bag and add B3 buffer-containing CDP-star (60 µL CDP-star/6 mL B3 buffer). Incubate 5 min at room temperature.
14. Take the filter from the solution, put on a paper towel for a few seconds to remove excess fluid, and wrap the filter in plastic film.
15. Expose to a photographic film for 5–30 min (see Note 18) and develop.

3.2.3. Selection of Single-Copy Plants

Single-copy plants are selected based on the data from the Southern blot analysis.

1. For each transformant, determine the number of border fragments obtained with the RB and LB probes.
2. For a single-copy plant with Ksc1- and Hsc1-derived T-DNA, only one RB/plant junction fragment and one LB/plant junction fragment are visualized. Select those plants for which only

one fragment was visible for the RB and LB probes. In case the Ksc2 T-DNA transformation vector was used, also select those plants with two RB/plant junction fragments and two LB/plant junction fragment (see Subheading 3.3).

- 3. To obtain homozygous seed stocks from these transgenic plants, the T1 plant is allowed to self-fertilize and the T2 stock is harvested. About 7–11 T2 plants are grown up, and the T3 seed stocks are obtained. Those seed stocks containing 100% transgenic plants are derived from homozygous T2 plants and can be used to propagate these transgenic lines (see Note 19).

3.3. Analysis of Inversion of the T-DNA Between the loxP Sites when Using the Ksc2 Transformation Vector

3.3.1. PCR Analysis of the Border Fragments

When the Ksc2 transformation vector is used, the CRE recombinase can invert the intervening transgene between the two *loxP* sites in the obtained resolved single-copy transformants, because, as mentioned, the transformants have two LB and two RB T-DNA/plant junction fragments on a Southern blot analysis. This flipping event can be confirmed by PCR analysis and prediction of the newly formed LB and RB T-DNA/plant junctions.

Four PCR reactions are needed for amplification of the original T-DNA and for the T-DNA with the inverted DNA segment between the *loxP* sites; more specifically, two PCR reactions for amplification of the original LB–neomycin phosphotransferase II (*NPTII*) and RB– β -glucuronidase (*GUS*) junctions and two PCR reactions for the newly formed LB–*GUS* and RB–*NPTII* fragments (see Fig. 2). Therefore, four primers have been designed (see Table 1): two border-specific primers that recognize a sequence before the border and the *loxP* sites (p1 and p4 for the LB and RB

Table 1
Primer list for the amplification of original and newly formed border fragments

Primer	Sequence	PCR product length (bp)
p1	5'CCTGGCCTAACATCACACCT3'	
p2	5'CACCCCCTGCTTTAATGAGA3'	
p3	5'CTGATAGCGCGTGACAAAAA3'	
p4	5'CAGGCCAAATTCGCTCTTAG3'	
p1 + p2		832
p1 + p3		1,219
p3 + p4		1,282
p2 + p4		994

region, respectively) and two primers specific for the selection marker (p2 in case of the *NPTII* gene as in the Ksc2 vector) and the expression cassette (p3 in case of the *GUS* gene as in the Ksc2 vector) (see Note 20).

1. Mix for each 25 μ L reaction (on ice) in a 0.2-mL tube:

10 $\times$ PCR buffer	2.5 μ L
Primer 1 (50 ng/ μ L)	2.5 μ L
Primer 2 (50 ng/ μ L)	2.5 μ L
MgCl ₂ (50 mM)	0.75 μ L
Taq polymerase (5 U/ μ L)	0.3 μ L
dNTP mix (2 mM)	2.5 μ L
DMSO (5%)	1.25 μ L
Plant DNA (100 ng)	$X\mu$ L
H ₂ O	12.7– $X\mu$ L

2. Prepare a control reaction without template DNA or plant DNA from a wild-type transformed plant.
3. Place tubes in a thermal cycler preheated to 94°C.
4. Run as follows: 5 min at 94°C; 30 cycles at 94°C for 45 s, at 55°C for 45 s, and at 72°C for 90 s; and for 5 min at 72°C.
5. Load 10 μ L of the PCR products supplemented with DNA loading buffer into the lanes of a 1% agarose gel.
6. After running and visualizing the PCR products (usually by EtBr or SYBR Safe), score the presence of the amplified products LB–*NPTII* (p1+p2), RB–*GUS* (p3+p4), LB–*GUS* (p1+p3), and RB–*NPTII* (p2+p4) (see Note 21).
7. The obtained PCR products can also be sequenced with primers p1 or p4 from the LB or the RB, respectively, to confirm the predicted sequence.

3.3.2. Prediction of the Size of the Restriction Fragments in the Newly Formed Border Fragments

The length of the newly formed LB and RB T-DNA/plant junctions should correspond to the predicted length as calculated from the size of the original LB–*NPTII* and RB–*GUS* fragments and from the distance between the restriction site(s) in the T-DNA and the *loxP* sequences. The predictions correspond to the extra bands on the DNA gel blot for the single-copy transformants with two hybridizing bands for the LB and RB T-DNA/plant junctions. Details of this calculation are described below and in Fig. 3.

1. Define the length of the two LB and RB fragments obtained with the LB and RB probes from the Southern blot results.
2. Identify one LB and one RB fragment with the largest difference in size (bp).

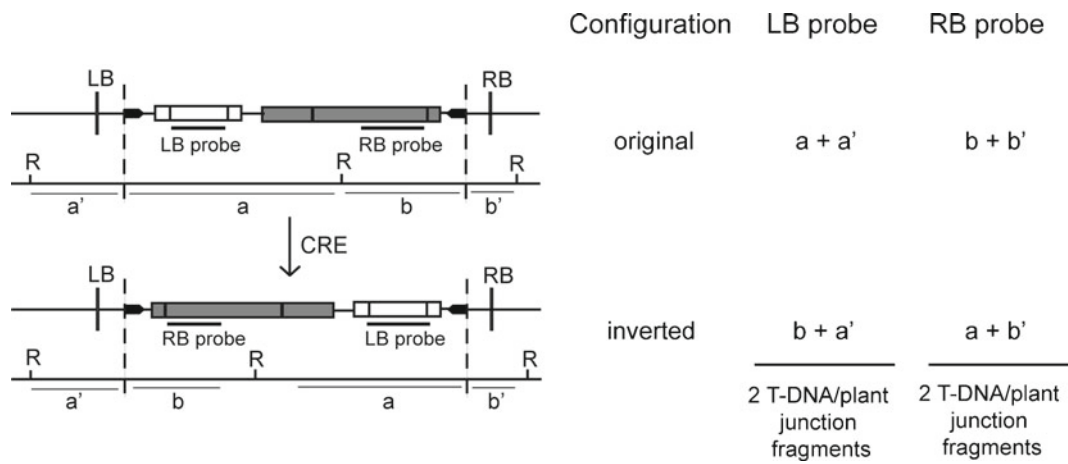


Fig. 3. Inversion of the *loxP*-flanked DNA fragment of the Ksc2 T-DNA. Schematic representation of the single-copy original integration structure and the inverted configuration allowing the detection of two different T-DNA configurations (not to scale). From the original single-copy configuration, one LB ($a + a'$) and one RB ($b + b'$) fragments will be observed. When the DNA between the two *loxP* sites is inverted, one additional border fragment consisting of the length of b and a' will be seen with the LB probe. Also, with the RB probe, a fragment with the sum of the sizes from a and b' will be present. *R* restriction site for the restriction enzyme used for the Southern analysis.

3. Determine the distance (in bp) between the used restriction site in the T-DNA vector and the *loxP* site at the LB and the RB.
4. Subtract these sizes from the size of the two identified fragments with the biggest difference in length. In this manner, the distance is known between the LB or RB borders and the nearest restriction site in the plant genome.
5. When inversion has occurred, the size of the newly formed border fragments is the sum of the identified length of the border to the nearest restriction site added to the length of the identified length of the inverted distance between the restriction site in the T-DNA vector and the *loxP* sites.
6. Also, the sum of both the LB and RB probes with the largest difference in size should be the same as the sum of the intervening LB and RB fragment sizes.

4. Notes

1. The selective agents are usually prepared as stock solutions with a 500- to 1,000-fold higher concentration than the end concentration in the growth medium. After filter sterilization, they are stored in a freezer (-20°C). For kanamycin, do not refreeze a defrosted sample because the solution is unstable.
2. Besides the Cre1 line in the C24 ecotype background, the CRE-expressing HSC6 line in the Col-0 ecotype background

(13) is available as well. This HSC6 line was obtained after floral-dip transformation of *Arabidopsis* (ecotype Columbia) with the HSC T-DNA (15). The *CRE* expression level in Cre1 is approximately twofold higher than that in the HSC6 line (15) as revealed by real-time PCR analysis.

3. As unique sites are present in the pKsc2, pKsc1, and Hsc1 plasmid (see Fig. 1), other gene expression cassettes can be inserted into these T-DNA transformation vectors.
4. The agrobacteria should be subcultured to fresh plates every 4 weeks for efficient growth and adequate cell density.
5. Use plants with inflorescences of 10–15 cm high and with immature flowers. The primary inflorescence can also be cut off to stimulate secondary inflorescences. Eventually, the plants can be grown under short-day conditions: 12 h light (21°C) and 12 h dark (18°C).
6. In our hands, the typical transformation efficiency for floral-dip transformation of Cre1 plants is in the range of 0.1–0.5%; in other words, 1–5 transformants are obtained from sowing approximately 1,000 seeds (25 mg).
7. Usually, an Aracon base and sheet is put immediately after dipping. In this manner, the dipped plants do not stick to each other because of the Silwet and cross contamination is avoided.
8. When the T-DNA does not contain a selection marker, T-DNA-containing seedlings can be selected by PCR analysis.
9. The “cold-snap” step is necessary because *Arabidopsis* seeds will preferentially germinate after experiencing a vernalization period.
10. Usually, we prepare DNA with the DNeasy Plant Mini Kit (Qiagen) from about 200 mg of *Arabidopsis* leaves.
11. With the unmodified Ksc2 and Ksc1 constructs, the *EcoRV* enzyme can be used, and for the Hsc1 construct, the *SphI* enzyme can be used to digest the DNA of the transformants, respectively.
12. Check in the T-DNA-containing plasmid whether the chosen restriction site(s) is (are) present at the predicted sites in the T-DNA.
13. We routinely use the SmartLadder because it is blotted and still visible after hybridization and exposure of the filter. Until now, this nonspecific hybridization of the SmartLadder has been seen with all our probes.
14. Blots can be stored when wrapped in plastic foils at 4°C after fixation.
15. In contrast to the hybridization method described previously (14), here, the nonradioactive detection with DIG-labeled

probes is presented. The reason is that the antibody used in the GeneImages CDP-star detection module is not available any more from the company (GE Healthcare). The DIG-based method works equally well in our hands and is done routinely with success. Optionally, a radioactive hybridization and detection method can be carried out.

16. Alternatively, a hybridization box and a water bath can be utilized.
17. The used probe can be kept in the hybridization solution in a 50-mL tube at -20°C and reused 3–4 times up to 1 year.
18. The membranes can be stripped and reused for hybridization, provided the membrane did not dry at any time throughout the procedure. For the stripping, wash the membrane 1 min in deionized H_2O , incubate the membrane twice for 15 min in 0.2 M NaOH supplemented with 0.1% SDS at 37°C . Afterward, rinse the membrane twice for 5 min in $2\times$ SSC and store in $2\times$ SSC.
19. If one wishes to remove the *CRE*-expressing transgene locus, the selected T1 transformants can be crossed to wild-type *Arabidopsis* plants, and hybrids will be obtained containing the T-DNA of interest and the CRE T-DNA in hemizygous condition. By selfing these hybrids, transgenics with the T-DNA of interest and without the CRE T-DNA can be obtained by PCR screening with a frequency of 25%.
20. When the T-DNA does not contain the kanamycin selection marker, another primer specific for another selection marker or the used gene expression cassette at the LB site of the T-DNA can be utilized instead of p2. The same holds true for p3.
21. When all four PCR products are amplified from the DNA of the analyzed transformant, then the T-DNA between the *loxP* sites has inverted. However, as a consequence of deletions of the T-DNA ends or rearrangements during the T-DNA integration, sometimes amplification of one or two border fragments can fail, but in that case, also the original border fragments will not be amplified. For information, p1 and p4 are situated 500 and 90 bp from the LB and RB, respectively.

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Chapter 27

A Developmentally Regulated Cre-*lox* System to Generate Marker-Free Transgenic *Brassica napus* Plants

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Abstract

In this chapter, a strategy for engineering marker-free *Brassica napus* plants is described. It is based on the Cre-*lox* site-specific recombination system and includes three essential steps. At first, the binary vector pLH-*nap-lx-cre-35S-bar-lx-vst* has been designed. In this vector, the *cre* gene and the *bar* expression cassette are flanked by two *lox* sites in direct orientation. The *lox*-flanked sequence is placed between a seed-specific napin promoter and a coding region for the *vstI* gene. At the second step, the *cre-bar* vector was transferred into *B. napus* hypocotyl explants by *Agrobacterium tumefaciens*-mediated transformation. Finally, T1 progeny was tested for excision of the marker gene at phenotypic and molecular levels. PCR, sequencing, and Southern blot analysis confirmed complete and precise deletion of the *lox*-flanked DNA region. This developmentally regulated Cre-*lox* system can be applied to remove undesirable DNA in transgenic plants propagated by seeds.

Key words: *Brassica napus*, Marker gene removal, Developmentally regulated site-specific recombination

1. Introduction

New *Brassica napus* varieties with improved agricultural, industrial, or nutritional traits were obtained via genetic transformation. The low efficiency of currently used DNA-delivery technologies results in a very small proportion of targeted plant cells actually integrating the recombinant DNA in the nuclear genome. Selectable marker genes, which are linked to the trait gene, allow the identification of transformed oilseed rape cells. These markers have, however, no function in the commercial transgenic crop. Potential benefits from marker gene removal include the reduction of risk assessment efforts, increased regulatory and market acceptance of

transgenic *B. napus*, and the possibility of retransformation using the same marker gene to insert additional traits. Promising methods have been developed for marker gene removal including cotransformation (1, 2), homologous recombination (3), and recombinase-mediated excision (4). Two technical approaches, namely, cotransformation (5, 6) and site-specific marker gene excision (7), have been described in the literature for *Brassicaceae*. The cotransformation method is generally based on the cointegration of the gene of interest and a selectable marker at unlinked loci followed by genetic segregation in the progeny. Two main factors can limit the application of this technique to oilseed rape: low cotransformation rate and insertion of the marker and trait genes into linked loci. The second alternative marker gene removal approach for *Brassicaceae* relies on using Cre-mediated site-specific recombination (8). The Cre-*lox* site-specific recombination system from bacteriophage P1 functions via interaction of a 38-kDa Cre recombinase protein with two 34 bp recognition *lox* sites (9). Cre-mediated recombination between two *lox* sites in direct orientation results in removing of the intervening DNA. According to the strategy used, the Cre protein can persist in *lox*-target plants for a long or a short period of time. In the first case, marker gene elimination includes the following steps. At first, *lox* target plants are generated and crossed with *cre*-harboring plants (10–12), or a second round of transformation with the *cre* construct (10, 12) is included. Marker-free plants can be selected in the T2 generation. This strategy is time and labor intensive and requires *cre* removal by genetic segregation. Alternatively, *cre* can be expressed in a transient or regulated manner. Transient expression methods include the provision of recombinase by *Agrobacterium tumefaciens* (13, 14) or viral vectors (15, 16). These vectors are mostly applicable to vegetatively propagated species. Combining regulated recombinase expression with autoexcision vectors might be the most reliable approach for marker gene elimination from oilseed rape. An autoexcision vector harbors a *lox*-embedded cassette including the recombinase gene linked to a selectable marker gene. *Cre* gene expression can be controlled by a chemically (17), heat shock (18) regulated or tissue-specific promoter (19). Several potential advantages result from this strategy. First, the time-limited recombinase expression allows a decrease in the possibility for negative effects of prolonged *cre* expression (20). Secondly, the marker gene and the recombinase gene are excised simultaneously saving time and effort, resulting from pollination/second transformation and segregation.

In the method described here, we exploited a developmentally activated *cre* gene to excise marker genes from oilseed rape. In the construct used, *cre* recombinase and *bar* expression cassette are flanked by two *lox* sites. *Cre* is placed under the control of the seed-specific napin promoter from *B. napus*. Tissue-specific recombinase

induction results in simultaneous removal of marker and *cre* sequences. The fact that we could introduce this construct into *B. napus* by *A. tumefaciens*-mediated transformation and select transgenic plants indicates that the *cre* gene was not expressed in somatic tissues during the selection step and that recombinase expression was restricted to specific tissues and time. We confirmed the marker-free status of T1 plants at phenotypical and molecular levels. The approach described here might be applicable for other generatively propagated species too.

2. Materials

2.1. Bacterial Strains

1. *Agrobacterium tumefaciens* strain AGLO (21).
2. *Escherichia coli* strain XL Blue.

2.1.1. Bacterial Medium and Additives

1. Luria–Bertrani (LB) medium: 10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride per liter.
2. Spectinomycin: 50 mg/mL stock solution in water. Store at -20°C .
3. Streptomycin: 50 mg/mL stock solution. Store at -20°C .
4. Ampicillin: 100 mg/mL stock solution. Store at -20°C .
5. Acetosyringone: 200 μM stock solution in DMSO. Store at -20°C (see Note 1).

2.2. Cloning the Binary Vector pLH-nap-lx-cre-35S-bar-lx-vst

1. Binary vector pLH7000 (22).
2. pUC and plx cloning vectors.
3. Restriction enzymes: *EcoRV*, *HindIII*, *KpnI*, *SaII*, *SacI*, *SmaI*, *XbaI*, *XhoI* (Fermentas), and *PacI* (NEB).
4. T4 DNA Ligase (Fermentas).
5. T4 DNA Polymerase (Fermentas).
6. Easy Pure DNA Purification Kit (Biozym).

2.3. *Agrobacterium tumefaciens*-Mediated *Brassica napus* Transformation

2.3.1. Plant Material

1. Mature seeds of *B. napus* (cv. Drakkar).
2. 5- to 7-day-old seedlings.

2.3.2. Growth Media and Additives

1. Basic MS medium (see Note 2).
2. Sucrose.
3. Agar.
4. Myo-Inositol.

5. MES.
6. Zeatin riboside: 4 mg/mL stock solution in 70% ethanol.
7. α -Naphthaleneacetic acid: 1 mg/mL stock solution in water. Store at -20°C .
8. 2,4-Dichlorophenoxyacetic acid (2,4-D): 2 mg/mL stock solution in DMSO. Store at -20°C .
9. 6-Benzylaminopurine (6-BAP): 1 mg/mL stock solution in water. Store at -20°C .
10. Phosphinothricin (PPT): 10 mg/mL stock solution in water. Store at -20°C (see Note 3).
11. AgNO_3 : 2 mg/mL stock solution in 70% ethanol. Store at -20°C in darkness.
12. Ticarcillin (TIC): 500 mg/mL stock solution in water. Store at -20°C .
13. Cefotaxime: 50 mg/mL stock solution in water. Light and temperature sensitive. Store at -20°C in darkness.
14. 2% (w/v) Sodium hypochlorite solution.

2.4. Basta Test of *Brassica napus* Plants

1. T1 progeny and wild-type *B. napus* seeds.
2. 0.05% Basta solution (Bayer, Germany).
3. Aatiram (Stähler, Germany).

2.5. Molecular Characterization of Target and Marker-Free Transgenic Plants

2.5.1. PCR Amplification

1. Genomic DNA from transgenic T0 and T1 and nontransgenic (wild-type) plants.
2. DNA polymerase and reaction buffer (Bioline).
3. dNTP mix (10 mM) (Bioline).
4. Oligonucleotide primers:
 - vst-e-forward, 5'-GGACATGAGCTACACATCACATATTA GCAT-3'
 - vst-e-reverse, 5'-AAGCTTGAAACATTCATTCACTTGGA GGTG-3'
 - vst-b-forward, 5'-TGCTGCGGGCGGCCGGCTTCAAGC ACGGGA-3'

2.5.2. Sequencing the PCR Recombination Products

1. PCR cloning vector (Promega's pGEM-Teasy vector).
2. Restriction endonucleases: *Eco*RI, *Sph*I, and *Sty*I (Fermentas).
3. T4 DNA Ligase (Promega).
4. Invisorb Spin Plasmid Mini Kit (Invitex).
5. Invisorb Fragment CleanUp Kit (Invitex).

2.5.3. Southern Blot Analysis

1. DNA Plant Maxi Kit (Qiagen).
2. Restriction endonucleases: *HindIII*, *MluI*, *NcoI*, *SmaI*, *SphI*, *StyI*, and *XhoI* (Fermentas).
3. AlkPhos Direct Labeling Kit (Amersham Pharmacia Biotech).
4. Primary wash buffer: 2 M Urea, 0.1% SDS, 50 mM Na phosphate (pH 7), 150 mM NaCl, 1 mM MgCl₂, and 0.2% blocking reagent.
5. Secondary wash buffer: 20× stock solution: 1 M Tris and 2 M NaCl, pH 10. Store up to 4 months in a refrigerator at 2–8°C.
6. Hybond-N⁺ nylon membrane (Amersham).
7. Detection reagent: CDP-Star (Boehringer, Mannheim, Germany).
8. X-ray film, X-ray cassette.
9. Depurination solution: 0.25 M NaCl.
10. Denaturation solution: 0.5 M NaOH and 1.5 M NaCl.
11. Neutralization solution: 1.5 M NaCl and 1 M Tris-HCl, pH 7.5.
12. 20× SSC: 3 M NaCl and 0.3 M trisodium citrate.

3. Methods

3.1. Cloning of the *pLH-nap-lx-cre-35S-bar-lx-vst* Binary Vector for *Brassica napus* Transformation

To design a *pLH-nap-lx-cre-35S-bar-lx-vst* vector, a number of fragments released from different plasmids are combined via multiple cloning steps. Figure 1 presents the detailed scheme of the cloning strategy. Perform each cloning step as follows (see Notes 4 and 5):

1. Digest both vector plasmid and plasmid containing the desired fragment/fragments with appropriate restriction enzymes according to the manufacturer's recommendations.
2. If necessary, perform a "blunt end" reaction by using T4 DNA Polymerase (Fermentas) according to the manufacturer's recommendations.
3. Resolve digests on 1% agarose gel and purify vector and insert using Easy Pure DNA Purification Kit (Biozym).
4. Set up the ligation reaction using T4 DNA ligase (Fermentas) and incubate the ligation reaction according to the guidelines for temperature and time.
5. Following the ligation reaction, transform *E. coli* cells and plate onto solid LB medium with 50 mg/L ampicillin for pUC, *plx*-based plasmids and 50 mg/L spectinomycin, and 20 mg/L

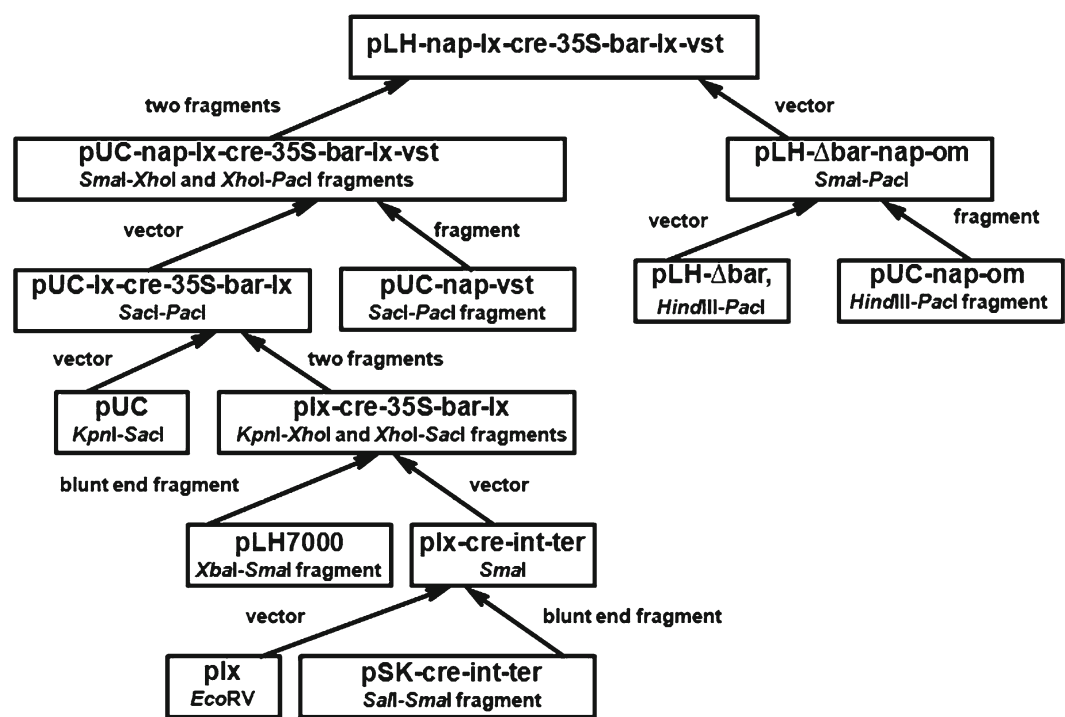


Fig. 1. A schematic overview of steps to clone the pLH-nap-lx-cre-35S-bar-lx-vst transformation vector.

streptomycin for pLH7000-based constructs. Incubate Petri dishes overnight at 37°C.

- 6. Select positive clones and confirm the successful ligation by digestion of DNA using restriction enzymes.
- 7. Transfer the final pLH-nap-lx-cre-35S-bar-lx-vst plasmid into *A. tumefaciens*.

3.2. Transformation of *Agrobacterium tumefaciens*

- 1. Transfect the pLH-nap-lx-cre-35S-bar-lx-vst construct into *A. tumefaciens* strain AGLO by modified freeze/thaw method (23) and select the transformants on solid LB medium supplemented with 300 mg/L streptomycin and 100 mg/L spectinomycin (see Note 6).
- 2. Incubate plates at 28°C for 2 days.
- 3. Isolate single colonies and inoculate into fresh liquid LB medium supplemented with appropriate antibiotics.
- 4. Verify the presence of the binary vector in *A. tumefaciens* by plasmid minipreparation and following restriction analysis.
- 5. Use a single *A. tumefaciens* colony harboring pLH-nap-lx-cre-35S-bar-lx-vst plasmid for *B. napus* transformation.

3.3. *Brassica napus* Transformation

Brassica napus transformation and regeneration is carried out according to Cardoza and Stewart (24) with some modifications.

The manipulations with seeds, explants, and regenerants should be performed using sterile tools and equipment in a laminar flow hood to avoid microbial contamination.

**3.3.1. Seed Sterilization
and Preparation
of Explants**

1. Sterilize seeds in 2% sodium hypochlorite for 30 min.
2. Wash the seeds with sterile distilled water for 10 min. Repeat this step five times.
3. Place seeds in sterile culture vessels containing MS medium and incubate 5–7 days under light conditions.
4. Excise hypocotyls; cut into segments of about 10–15 mm using forceps and scalpel (see Note 7).
5. Place the freshly prepared hypocotyl explants in 15-cm Petri dishes with liquid MS medium.

3.3.2. *Agrobacterium tumefaciens* Culture

1. Streak out bacterial stock (AGLO harboring the pLH-*nap-lx-cre-35S-bar-lx-vst* construct) onto LB agar medium containing 20 mg/L streptomycin and 50 mg/L spectinomycin and incubate plates overnight at 28°C (see Note 8).
2. Pick up and culture a single colony in 100 mL liquid LB medium supplemented with selection antibiotics and 100 µM acetosyringone and cultivate at 28°C at 180 rpm overnight.
3. Centrifuge at 3,000×*g* for 15 min and resuspend the pellet with liquid MS medium to final OD₆₀₀ 0.6–0.8.
4. Use this solution to transform *B. napus* hypocotyl explants.

**3.3.3. Transformation
of *Brassica napus*
Hypocotyl Explants:
Inoculation and
Cocultivation**

1. Incubate explants in *A. tumefaciens* suspension with shaking for 30 min.
2. Collect hypocotyl explants using forceps and blot dry on sterile filter paper to remove excess of bacterial suspension.
3. Place the explants on Petri dishes containing MS medium without growth regulators, antibiotics, and selection agents.
4. Incubate in the dark at 26°C for 2 days.
5. Collect explants into sterile cultivation vessels using forceps.
6. Wash explants three times with liquid MS medium supplemented with 500 mg/L (TIC) and 50 mg/L cefotaxime for 20 min to inhibit the growth of *A. tumefaciens*.

**3.3.4. Transformation
of *Brassica napus*
Hypocotyl Explants:
Selection
and Regeneration
of Transgenic Plants**

1. Transfer explants into MS medium with 2 mg/L 2,4-D, 10 mg/L AgNO₃, 500 mg/L TIC, and 5 mg/L PPT to initiate callus formation. Cultivate 2–3 weeks (see Notes 9 and 10).
2. Transfer hypocotyls with callus into MS medium supplemented with 4 mg/L BAP, 2 mg/L ZR, 500 mg/L TIC, 5 mg/L AgNO₃, and 5 mg/L PPT to promote organogenesis.

- Following 2 weeks subculture, the explants are transferred into MS medium containing 3 mg/L BAP, 2 mg/L ZR, 5 mg/L PPT, 5 mg/L AgNO₃, and 500 mg/L TIC.
3. After shoot initiation, the explants are transferred into MS medium supplemented with 0.05 mg/L BAP, 5 mg/L PPT, 5 mg/L AgNO₃, and 500 mg/L TIC for shoot elongation.
 4. Cut regenerated shoots and cultivate them on MS solid medium supplemented with 0.5 mg/L α -Naphthaleneacetic acid, 3 mg/L PPT, and 300 mg/L TIC for rooting and recovering complete plants.
 5. All the above media contain 30 g/L sucrose, 300 mg/L *myo*-inositol, and 500 mg/L MES, pH 5.6–5.8. Solid medium is solidified with 7 g/L agarose. The cultures are maintained at 24°C under 16-/8-h light conditions.
 6. The rooted plants are transferred to soil and self-pollinated (see Notes 11 and 12).

3.4. Basta Germination Test

1. Put T1 seeds into 15-cm Petri dishes, soak with 0.05% aqueous Basta solution, and cultivate at 20°C and 18/6 h photoperiod (see Note 13).
2. Add new Basta aliquots every 3–4 days.
3. Score the seedlings for resistance after 12 days of cultivation. The resistant plants possess expanded green cotyledons and elongated hypocotyls and roots, whereas sensitive plants exhibit yellow cotyledons and poorly developed hypocotyls and roots (Fig. 2).



Fig. 2. Basta test of T1 progeny plants. T1 seeds of lines vst-9, vst-46, and vst-61 were grown in the presence of 0.05% Basta solution for 12 days. Control (vst-9, c) was germinated without herbicide.

3.5. Molecular Characterization of Transgenic Plants

3.5.1. PCR Analysis

The marker gene elimination is determined by using two sets of primers, one (vst-e-forw and vst-e-rev) anneals to the 3' of the napin promoter and 5' of the *vstI* gene and the other (vst-b-forw and vst-e-rev) to the 3' end of the *bar* expression cassette and the 5' of the *vstI* gene. The first set of primers amplifies a 4,070-bp fragment from the original pLH-*nap-lx-cre-35S-bar-lx-vst* T-DNA or a 687-bp fragment from the recombined DNA as illustrated in Fig. 3a, b. The second set of primers detects a 800-bp product from parental T-DNA. In plants with complete *bar* gene excision, this amplification product is absent (Fig. 3a).

1. Extract DNA from leaf tissue of parental T1 and wild-type plants using the CTAB method. A detailed description of this method can be found in ref. 4.
2. Prepare PCR reaction mixture for 25 μ L: 40 ng template DNA, 2.5 μ L of 10 $\times$ PCR buffer, 15 pmol of each primer, 1 μ L of 10 μ M dNTPs, and 0.5 U of Taq polymerase. Include wild-type, parental plant, and plasmid DNA controls.
3. Perform PCR reaction with initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 30 cycles of denaturation at 94 $^{\circ}$ C for 1 min,

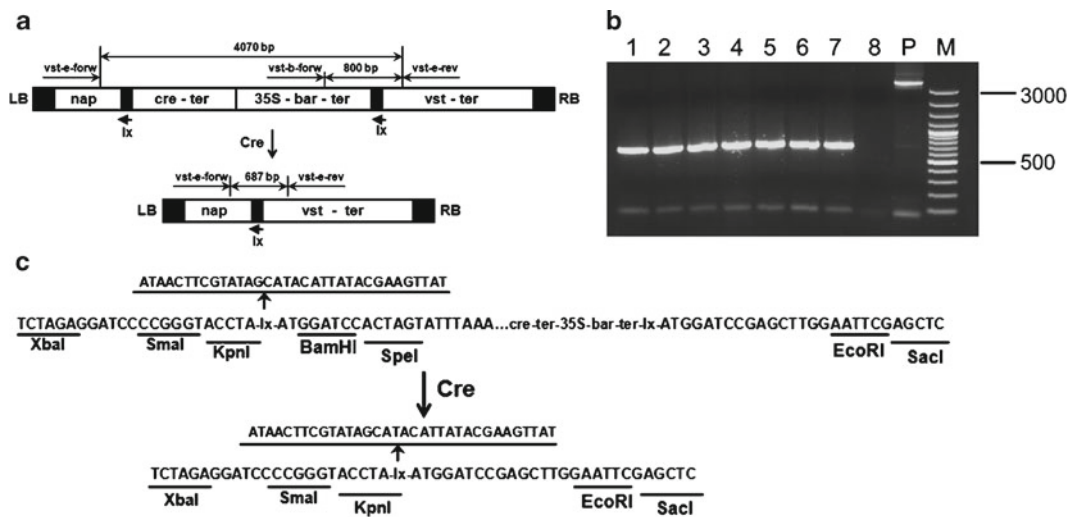


Fig. 3. Molecular analysis of cre-mediated *bar* gene excision. (a) Schematic representation of the T-DNA region of pLH-*nap-vst-lx-35S-bar-lx* before and after site-specific marker gene elimination. Primers vst-e-forw, vst-b-forw, and vst-e-rev are used to select marker-free plants. Primer combination vst-e-forw/vst-e-rev amplifies a 4,070- or a 687-bp fragment from intact or recombined T-DNA. The second combination vst-b-forw/vst-e-rev allows detecting plants with incomplete *bar* gene excision. In case of complete excision, no product is generated, whereas a 800-bp fragment is amplified from non-recombined pLH-*nap-vst-lx-35S-bar-lx* T-DNA. (b) PCR analysis. Genomic DNA extracted from T1 progeny plants is investigated using vst-e-forw and vst-e-rev primers. Lanes 1–8 indicate vst-9, vst-21, vst-38, vst-46, vst-47, vst-48, and vst-61 lines and wild-type *B. napus* plant, respectively. P, PCR fragment derived from pLH-*nap-vst-lx-35S-bar-lx* plasmid DNA. M molecular marker, 100 bp (Fermentas). (c) Comparison of sequences surrounding *lox* sites in parental (upper sequence) and recombined (lower sequence) T-DNA. The recombined sequence is derived from a 687-bp PCR product. PCR products from vst-9, vst-38, and vst-46 lines were investigated. Restriction sites characteristic to intact pLH-*nap-vst-lx-35S-bar-lx* DNA and rejoined *lox* site are presented.

annealing at 60°C for 1 min and extension at 72°C for 1 min. Maintain the reaction mixture at 72°C for 10 min before completion.

4. Analyse the PCR-amplified products in agarose gel using appropriate molecular marker (see Note 14).

3.5.2. Sequencing of the PCR-Amplified Recombination Product

1. Isolate genomic DNA from T1 progeny plants using CTAB method.
2. Prepare PCR reaction for 50 µL: 1 µL of genomic DNA (~100 ng), 5 µL of 10× PCR buffer, 15 pmol of each primer, 1.5 mL of 10 µM dNTPs, and 1–2.5 U of Taq polymerase. Adjust final volume with sterile distilled water.
3. Set up the PCR reaction as described in Subheading 3.5.1.
4. Examine the PCR product by running a small aliquot in an agarose gel by electrophoresis and purify the remaining product using Invisorb Fragment CleanUp Kit (Invitex) as described in product manual.
5. Set up ligation reaction of PCR-amplified 687-bp DNA fragment with pGEM-Teasy cloning vector following manufacturer's protocol.
6. Take 5 µL of the ligation mixture and transform into competent *E. coli* cells.
7. Screen the recombinant clones using blue/white selection procedure following the manufacturer's protocol and isolate the recombinant plasmid DNA using Invisorb Spin Plasmid Mini Kit (Invitex).
8. Digest the pGEM-RP vector containing the PCR recombination product with *SphI*-*StyI* to verify the DNA fragment.
9. Sequence correct clones.

The parental *lox* sites are linked to characteristic restriction sites, the left to *XbaI*, *SmaI*, and *KpnI* and the right to *EcoRI* and *SacI* (Fig. 3c). The position of these restriction sites in the rejoined *nap-lx-rsI* sequence confirms that cre-mediated *bar* gene excision is precise.

3.5.3. Southern Blot Analysis

The site-specific marker gene removal and the absence of the *bar* selectable marker gene can be ascertained by Southern blot analysis of the genomic DNA. Digest genomic DNA extracted from transgenic and wild-type *B. napus* plants with appropriate restriction enzymes and hybridize with a labeled probe. Perform the following steps carefully.

3.5.4. Southern Blot Analysis: DNA Transfer to Membrane

1. Extract genomic DNA from transgenic as well as from wild-type plants using DNeasy Plant Maxi Kit (Qiagen) as described in the product manual.

2. Digest 15–18 μ g DNA with appropriate restriction enzymes overnight at the prescribed temperature depending on the enzyme. Include wild-type DNA as a control (see Note 15).
3. At the end of the digestion, add the appropriate amount of gel-loading buffer and separate fragments of DNA by electrophoresis through a 0.8% agarose gel.
4. Depurinate the DNA by soaking in 0.25 N HCl for 10 min and rinse three times with distilled water for 5 min each.
5. Denature DNA by soaking the gel in denaturation solution for 30 min and rinse three times with distilled water for 5 min each.
6. Neutralize DNA by soaking the gel in neutralization solution for 30 min and rinse three times with distilled water for 5 min each.
7. While the gel is soaking in the neutralization solution, cut a piece of membrane, prewet in water, and then immerse in 20 $\times$ SSC for at least 5 min. Use gloves and blunt-ended forceps to handle the membrane.
8. Remove the gel from the neutralization solution, invert it so that the smooth surface faces the nylon membrane and place it on the support covered with wet filter paper.
9. Place the membrane on top of the gel and cover it with three pieces of wet filter paper and a stack of paper towels. Put a glass plate on the top of the stack and apply pressure by placing a weight of 300–500 g. Allow the DNA to transfer overnight.
10. After blotting, carefully dismantle the transfer apparatus, remove the membrane with transferred DNA, and fix the nucleic acid to the membrane by using an UV cross-linking procedure.

3.5.5. Southern Blot Analysis: Preparation of the Labeled Probe

1. Release the probes from the plasmids by digesting with appropriate restriction enzymes.
The following probes are used in Southern blots: For confirming the removal of the *bar* marker gene, a 570-bp probe is obtained after restriction of the pLH-*nap-lx-cre-35S-bar-lx-vst* with *Xho*I-*Mlu*I; for detection of site-specific recombination events, a 323-bp probe is received after digestion of pGEM-Teasy-RP with *Sph*I-*Sty*I. A detailed protocol for obtaining the pGEM-Teasy-RP plasmid can be found in ref. 25.
2. Resolve the digest on agarose gel and purify the fragments using Easy Pure DNA Purification Kit (Biozym).
3. Prepare probes by Alkphos Direct Labeling Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

3.5.6. Southern Blot Analysis: Hybridization and Detection

1. Place the blot in sufficient volume of Alkphos Direct hybridization buffer (Amersham Pharmacia Biotech) and prehybridize for 1.5–2 h at 57°C in the hybridization oven.

2. Add the labeled probe to the buffer used for the prehybridization step and hybridize at 57°C overnight.
3. After overnight hybridization, discard the hybridization buffer with the labeled probe and wash the membrane with primary wash buffer three times at 57°C for 10 min each. Perform a further wash in fresh secondary wash buffer twice at room temperature for 10 min each.
4. Remove the membrane from the secondary wash buffer, pipette detection reagent CDP-Star (Boehringer) onto it, and leave for 2–5 min.
5. Wrap the blot in Saran Wrap and place it, DNA side up, in the film cassette.
6. Expose X-ray film to the hybridized membrane in the dark.
7. Remove the film and develop it. If required, expose a second film for an appropriate length of time (see Note 16).

Hybridization with a 323-bp probe specific for site-specific recombination to *Hind*III digested genomic DNA of T1 plants yields a 2,776-bp fragment after correct gene excision of the *lox*-flanked region after. For the parental line, no hybridization signal is observed (Fig. 4a, b). A 6,074-bp fragment characteristic for the

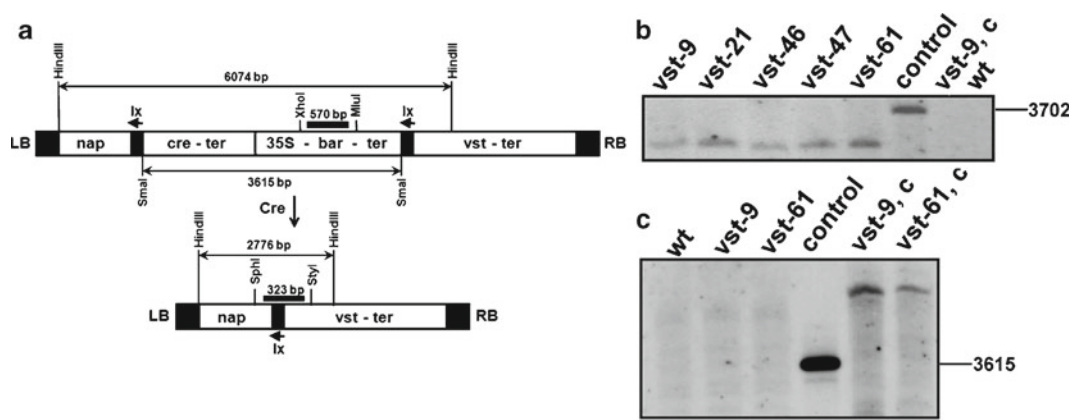


Fig. 4. Southern blot analysis to confirm the site-specific marker gene excision in *Brassica napus*. (a) Schematic representation of hybridization fragments and probe-binding sites. A 570-bp *Xho*I-*MLu*I bar probe detects a 6,074-bp *Hind*III fragment from intact pLH-*nap-vst-lx-35S-bar-lx* plasmid and no fragment after marker gene excision. A 323-bp *Sph*I-*Sty*I probe yields a 2,776-bp *Hind*III hybridization product and no product from parental lines. Genomic DNA of T1 progeny plants verified as marker free using vst-e-forw, vst-b-forw, and vst-e-rev primers is digested with *Hind*III and hybridized with probes specific for *nap-lx-vst* rejoined sequence (b) and *bar* gene (c). (b) Genomic DNA was extracted from parental and T1 progeny plants, cleaved with *Hind*III and probed with probe specific for site-specific recombination. Lanes Vst-9, vst-21, vst-46, vst-47, and vst-61 represent T1 progeny plants. DNA of parental Vst-9, wild-type *B. napus* plant, and plasmid pGEM-RP cleaved with *Nco*I is used as control. (c) Genomic DNA was extracted from T1 progeny plants confirmed as marker free by Southern blot and parental plants, restricted with *Hind*III, and hybridized with a *bar* probe. Lanes vst-9 and vst-61 show T1 progeny plants. Parental vst-9, vst-61, wild-type *B. napus* plants, and a 3,615-bp *Sma*I fragment of pLH-*nap-vst-lx-35S-bar-lx* plasmid are used as controls.

intact, nonrecombined construct is observed after hybridization of a 570-bp *bar* probe with genomic DNA extracted from parental plants. Absence of this fragment in T1 transgenic plants confirms the complete marker gene excision (Fig. 4a, c).

4. Notes

1. Application of 100 μ M acetosyringone in LB and MS cocultivation medium can improve the transformation frequency. Acetosyringone can oxidize over time and lose its activity. Therefore, store the crystalline chemical and the 200 μ M stock solution in dimethylsulfoxide (DMSO) at -20°C in the dark.
2. The ingredients for basic MS medium can be supplied as ready-mixed powders from many supplies (e.g., Duchefa, Gibco-BRL, Sigma). For preparing the medium, the use of ready-mixed powders is very convenient to obtain reproducible results.
3. The type of selective agent used in the medium is determined by the marker gene in the pLH-*nap-lx-cre-35S-bar-lx-vst* construct. In our study PPT, selection is used with the *bar* marker from *Streptomyces hygroscopicus*, which encodes a PPT acetyltransferase.
4. To carry out cloning, an intron sequence should be introduced into the *cre* gene. The intron sequence prevents *cre* recombinase expression in bacterial cells and, therefore, undesired recombination.
5. The large size of pLH7000-based plasmid may increase the difficulty to clone into it directly. Therefore, it is also possible to use a smaller pUC vector for cloning the pUC-*lx-cre-35S-bar-lx-vst* plasmid with subsequent subcloning into pLH7000. To do this, follow the cloning scheme depicted in Fig. 1 and use *Sma*I, *Xho*I, and *Pac*I restriction sites.
6. The binary plasmid pLH-*nap-lx-cre-35S-bar-lx-vst* can be introduced into *A. tumefaciens* by the freeze/thaw as well as by the electroporation method.
7. When setting up a large experiment, change the scalpel blades frequently. Flame sterilizing will blunt the blade over time. The cut surface of the explants is the target tissue for transformation and regeneration. Damaged hypocotyl tissue does not respond well in culture.
8. It is important that the *A. tumefaciens* culture is fresh and not taken from an older plate. We routinely use bacteria freshly streaked from a stock frozen at -80°C for each transformation.

9. The seed-specific napin promoter from *B. napus* has been selected for tissue-specific expression of the *cre* recombinase. Care must be taken because some developmentally regulated promoters may be active in somatic tissues resulting in low transformation efficiency.
10. The heat-sensitive components (hormones and antibiotics) must be sterile filtered and added into autoclaved media cooled to 50°C.
11. After the transfer to the soil, the plants from in vitro culture are very sensitive to normal humidity and therefore must be covered with transparent plastic bags to maintain high humidity. The bags can be removed after 2–3 weeks to expose the plants to normal environment.
12. When the flowering commences, bags should be placed over inflorescences until seed formation and maturation to prevent cross-pollination.
13. Treatment of the seeds with 3 mg/g of Aatiram (Stähler, Germany) is highly recommended to protect plants against seed-born fungi.
14. The *vst-e-forw* and *vst-e-rev* primers used to select recombination events are specific to 3' end of the napin promoter and 5' end of the *vstI* gene. Such design allows amplifying a 687-bp fragment from the recombined *nap-lx-vst* sequence. In general, primers should be designed which bind to the left and right sequences from the rejoined *lox* site.
15. It is important to check the digestion efficiency of the genomic DNA before transferring to membrane. Partial or incomplete digestion will hinder the transfer of DNA to the membrane and produce misleading results.
16. For initial experiments, try a 1-h exposure. The signal reaches a peak ~3–4 h after addition of the detection reagent. Subsequent exposures can be made with suitably adjusted exposure times to get optimum signal-to-noise ratio.

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Exploiting MultiSite Gateway and pENFRUIT Plasmid Collection for Fruit Genetic Engineering

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Abstract

MultiSite Gateway cloning techniques based on homologous recombination facilitate the combinatorial assembly of basic genetic pieces (i.e., promoters, CDS, and terminators) into gene expression or gene silencing cassettes. pENFRUIT is a collection of MultiSite Triple Gateway Entry vectors dedicated to genetic engineering in fruits. It comprises a number of fruit-operating promoters as well as C-terminal tags adapted to the Gateway standard. In this way, flanking regulatory/labeling sequences can be easily Gateway-assembled with a given gene of interest for its ectopic expression or silencing in fruits. The resulting gene constructs can be analyzed in stable transgenic plants or in transient expression assays, the latter allowing fast testing of the increasing number of combinations arising from MultiSite methodology. A detailed description of the use of MultiSite cloning methodology for the assembly of pENFRUIT elements is presented.

Key words: Gateway cloning, Gene expression, Gene silencing, HpRNAi, Transient expression, Agroinjection

1. Introduction

Fruits are natural factories of edible, health-promoting substances. Increasing the concentration and/or broadening the spectrum of added-value compounds produced in fruits is a challenging biotechnological goal. In fruit biotechnology, a good experimental design includes testing various expression cassettes controlled by different promoters, which should be active at a given development phase or tissue (1–3). The Gateway cloning system, based on homologous recombination (4), has become a popular alternative to traditional cloning methods based on the T4 ligase for constructing expression cassettes, and thus several Destination vector collections for plants are currently available to the scientific community (5–7). MultiSite Gateway system is a variation that enables to construct in

a vector up to four fragments “in tandem” by using a single recombination reaction. In this way, a collection of vectors can be developed and combined with a particular gene of interest (GOI) (8–10). Entry vector collections are designed to facilitate the combinatorial assembly of basic genetic pieces (i.e., promoters, CDS, and terminators) into gene expression or gene silencing cassettes. pENFRUIT is an Entry vector collection specifically dedicated to genetic engineering in fruits and made available for the research community through a plasmid repository (8). pENFRUIT collection comprises a number of fruit-operating promoters covering a wide range of tissues and developmental stages, as well as a number of C-terminal tags for the labeling of the protein of interest. Altogether, pENFRUIT provides a total of 78 possible combinations for the expression of a GOI in the tomato fruit. Moreover, pENFRUIT vectors can be used for hpRNAi strategies with the sole requirement of a traditional cloning step using restriction enzymes.

Concomitantly with the development of increasingly versatile cloning methods, the need for simple and rapid analysis of gene function has also increased, so the use of transient expression assays has bloomed as an alternative to the analysis of stable transformants (11–13). Transient expression allows the production of recombinant proteins in a short time and does not require the use of a marker gene for selecting transformed plants. This results in smaller Expression vectors with more efficient ligation reactions and bacterial transformation. Agroinjection is a transient methodology which directs transient expression of foreign genes directly in the fruit (11). Injection of diluted *Agrobacterium* cultures at different immature stages resulted in transient expression of reporter genes in placenta, gel, and inner pericarp layers.

In this chapter, we describe in detail the methodology employed for the construction of new gene expression cassettes operating in the fruit using Gateway-based pENFRUIT vector collection. Furthermore, the testing of selected constructs in fruits using transient expression (agroinjection) is also described.

2. Materials

2.1. attB4r/attB3r GOI PCR Amplification and BP Cloning

1. HPLC- or PAGE-purified attB4r and attB3r flanking oligonucleotides (Invitrogen, Carlsbad, CA, USA). Advantage HD Polymerase Mix (Clontech, Mountain View, CA, USA). dNTP mixture (10 mM each dNTP) and sterilized water.
2. For gel extraction and purification: 1% agarose TAE 1× (40 mM Tris–acetate and 1 mM Na₂EDTA). For polyethylene glycol (PEG) purification: 1× TE buffer, pH 8.0 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Thirty percent PEG 8000/30 mM

MgCl₂ supplied with the BP Clonase™ II Enzyme Mix Kit (Invitrogen) and a microcentrifuge (Micro Centaur, SANYO).

3. MultiSite Gateway® Pro pDONR221 P4r-P3r (Invitrogen) vector for each *attB4r-/attB3r*-flanked PCR product. BP Clonase™ II Enzyme Mix Kit (Invitrogen). 1× Tris–EDTA (TE) buffer, pH 8.0 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Proteinase K solution (2 µg/µL) as included in the BP Clonase Kit (Invitrogen).
4. A Thermolyne Dri-Bath Model 17600 (Thermo Fisher Scientific Inc., Hampton, USA) thermoblock or a water bath (Precistern S-385) set at 37°C.

2.2. Cloning of the GOI in pEFS4r-3r and pEFS3-2 Vectors

1. DNA containing your GOI sequence and pEFS4r-3r and pEFS3-2 vectors from Addgene plasmid repository (<http://www.addgene.org/pgvecl>). Two different restriction enzymes recognizing the polylinker sites *SacI*, *SmaI*, or *XhoI* and their buffers (New England Biolabs, Hitchin, UK).
2. T4 DNA ligase (Invitrogen) and a thermoblock set at 24, 37, and 65°C.

2.3. *Escherichia coli* Cell Transformation and Culture

1. One Shot® TOP10 or One Shot® Mach1™ T1R chemically competent *Escherichia coli* kit (Invitrogen). Sterile LB liquid medium and solid LB agar (LBA) plates containing 50 µg/mL kanamycin in case of Entry clones (BP reaction) or 50 µg/mL spectinomycin for Expression clones (LR reaction).
2. E.Z.N.A.® Plasmid Mini Kit II (Omega Bio-tek, Doraville, GA, USA). A 42°C water bath (Precistern S-385) and 37°C shaking incubators.

2.4. Triple Gateway LR Recombination Reaction

1. LR Clonase™ II Plus Enzyme Mix Kit (Invitrogen). Keep at –20 or –80°C until immediately before use.
2. Miniprep-purified plasmid DNA of Entry clones pEF1-4, pEF4r-3r (or pEFS4r-3r), and pEF3-2 (or pEFS3-2) (supercoiled, 10 fmol). Twenty fmoles of purified plasmid DNA of pKGW,0 (Plant Systems Biology, Ghent, Belgium) for GOI expression or 20 fmol of supercoiled pDest/EFS Destination vector for GOI silencing.
3. 1× Tris–EDTA (TE) buffer, pH 8.0 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), Proteinase K solution (2 µg/µL) provided by the LR Clonase Kit (Invitrogen). Thermoblock or a water bath (Precistern S-385) set at 37°C.

2.5. *Agrobacterium tumefaciens* C58 Transformation and Agrobacterium Injection

1. Eppendorf tubes containing 40 µL of electrocompetent *Agrobacterium* C58 cells stored at –80°C. Electroporator (Bio-Rad, gene pulser 165-2077), 1-mm electroporation cuvettes (Bio-Rad Laboratories, CA, USA), and 15-mL tubes

containing 250 μL of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , and 20 mM glucose). Sterile LB liquid medium and solid LB agar (LBA) plates containing 50 $\mu\text{g}/\text{mL}$ spectinomycin and 50 $\mu\text{g}/\text{mL}$ rifampicin antibiotics and a growing chamber set at 28°C. For *Agrobacterium* DNA minipreps, use QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA, USA).

2. *Agrobacterium* cultures and subcultures' mediums for agroinjection: 15-mL tubes containing 3 mL of YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, and pH 7.4), 2 mM MgSO_4 , and spectinomycin and rifampicin (50 $\mu\text{g}/\text{mL}$ each) antibiotics. A shaker and a growing chamber set at 28°C.
3. Infiltration MES buffer: 10 mM MES (Sigma-Aldrich, MO, USA), 10 mM MgCl_2 , and 200 μM acetosyringone (Sigma-Aldrich). To prepare the 200-mM stock solution, dissolve 390 mg acetosyringone in 10 mL of dimethyl sulfoxide. Filter-sterilize and store at -20°C. Spectrophotometer (UV/VIS Spectrophotometer SP8001, Dinko) set at a wavelength of 600 nm and transparent plastic cuvettes.
4. Tomato fruits (*Solanum lycopersicum* cv. Micro Tom) at a developing green stage 25 days postanthesis (25 dpa) or in mature green (30–35 dpa), and sterile 1-mL Plastipak syringes with needle.

2.6. Fluorescence-Based Transient Expression Analysis

1. Tomato fruits (*Solanum lycopersicum* cv. Micro Tom) agroinjected with *Agrobacterium* strains containing fluorescent protein constructs.
2. A Typhoon Trio Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA) fluorescence scanner (8610 or 9210 Typhoon model) with gel alignment guides and ImageQuant Tools software. A clean low-fluorescent glass plate, powder-free gloves, and a meat cutter (CF-172 Fagor, Spain).

3. Methods

For our expression assays, we have used the pENFRUIT collection vectors which are based on the three-fragment MultiSite Gateway PRO system and are publicly available in the Addgene repository (<http://www.addgene.org/pgvec1>) access numbers 20083–20107. The pENFRUIT collection is composed of three subcollections of input vectors: (a) pEF1-PROM-4 containing promoter regions, (b) pEF4r-GOI-3r containing reporter genes, and (c) pEF3-TER-2 containing terminators and 3' marking sequences (8). For the triple recombination, the pKGW,0 binary plasmid (Plant Systems Biology) is used as Destination vector.

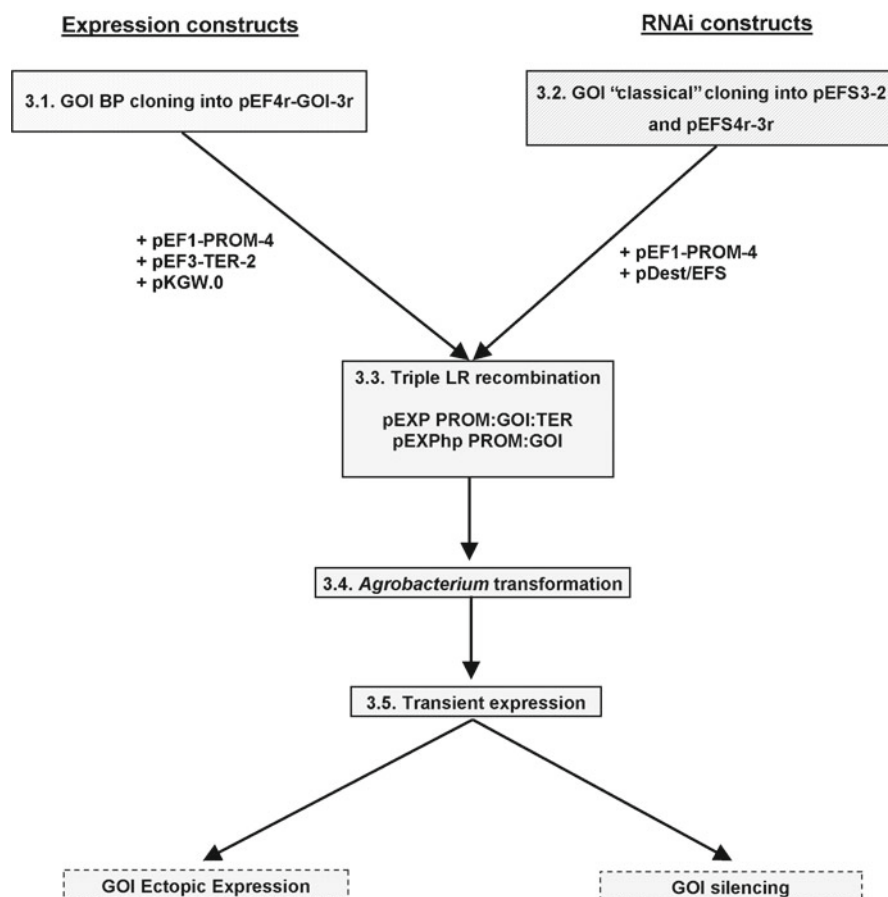


Fig. 1. Scheme representing the necessary steps for the expression and the silencing of a GOI. GOI expression requires, as a previous step, the making of pEF4r-GOI-3r Entry vector by BP recombination, while the GOI silencing requires the classic cloning of a GOI fragment in the pEFS4r-3r and pEFS3-2 vectors. These steps are prior to LR recombination reaction which, in case of the GOI expression, requires Entry vectors containing the promoter and terminator sequences (pEF1-PROM-4 and pEF3-TER-2) and the pKGW,0 Destination vector. For the GOI silencing, pEFS1-PROM-4 Entry vector adding the promoter sequence, and the pDest/EFS Destination vector are needed. The viability of the resulting Expression vectors is checked by transient expression in tomato fruits (see Subheading 3.5) using the agroinjection technique.

In the case of hpRNAi strategies, a fragment of the GOI is cloned into pEFS4r-3r and pEFS3-2 vectors using two of the restriction enzymes that cut in the polylinker region (*Sac*I, *Sma*I, and *Xho*I sites), which is set in reversed orientations in each plasmid. The GOI fragment is, in this way, inserted in inverted orientations in separate plasmids. A scheme of the strategies for a parallel ectopic expression and/or RNAi silencing of a GOI is shown in Fig. 1.

3.1. BP Cloning of a GOI for Its Expression in Fruits: Construction of a pEF4r-GOI-3r Plasmid

For the construction of a pEF4r-GOI-3r Entry vector, the GOI has to be amplified with oligonucleotide primers containing *att*B4r/*att*B3r recombination sites and incorporated into pDONR 221 P4r-P3r by BP-mediated recombination.

1. Design forward and reverse oligonucleotides for your GOI template including 18–24 bp gene-specific sequences plus the sequences corresponding to *attB*4r (Fwd 5'-GGGG ACA ACT TTT CTA TAC AAA GTT GNN) and *attB*3r (Rev 5'-GGGG AC AAC TTT ATT ATA CAA AGT TGT) recombination sites (see Note 1).
2. Amplify your GOI sequence by PCR reaction using Advantage HD Polymerase Mix (Clontech) which is suitable for producing high fidelity and long amplicon size (until 8.5 kb).
3. Purify the amplified GOI by electrophoresis through a 1% agarose gel. Prepare a thick TAE 1× minigel and charge the entire PCR reaction (50 µL) in a single well. Purify the DNA using QIAquick Gel Extraction Kit (QIAGEN) obtaining 50 µL of elution volume. For large or difficult-to-clone GOI fragments, the use of ethidium bromide and UV light should be avoided to reduce DNA fragmentation. Quantify the DNA by using a Nanodrop spectrophotometer (Nanodrop ND-100 Spectrophotometer, Thermo Fisher Scientific Inc.). Alternatively, a PEG purification protocol is suggested by Invitrogen to purify *attB* PCR products (see Note 2).
4. Put 15–150 ng of your *attB*4r-/*attB*3r-purified GOI in a tube; add 1 µL of pDONR vector (pDONR 221 P4r-P3r, 150 ng) and TE buffer to reach a total volume of 8 µL. Remove BP Clonase II enzyme (Invitrogen) from –20°C; thaw on ice for 2 min and mix briefly with the vortex. Add 2 µL of the enzyme to the BP reaction tube, mix by vortexing, and incubate BP reaction tube at 25°C for 1–16 h using a thermoblock (Thermolyne Dri-Bath Model 17600) (see Note 3). To stop the reaction, add 1 µL of Proteinase K solution and incubate the tube at 37°C for 10 min.
5. Take a tube containing chemically competent *E. coli* cells, One Shot Mach1 T1R competent cells (Invitrogen), from –80°C and thaw them on ice. Add 2 µL of BP reaction, shaking the contents gently, and perform transformation following manufacturer's instructions. Collect 50–150 µL from the bacterial culture and spread in LB plates containing 50 µg/mL kanamycin. Incubate the plates in a 37°C growing chamber for 16 h.
6. Pick 5–10 colonies using sterilized toothpicks, grow them overnight in liquid media, and obtain DNA minipreps from them using the E.Z.N.A.® Plasmid Mini Kit II (Omega Bio-tek). Confirm the pEF4r-GOI-3r Entry vector by restriction analysis or by sequencing using M13 forward and reverse primers.

3.2. "Classical" Cloning of the GOI for Silencing Strategies: Construction of pEFS4r-GOI-3r and pEFS3-IOG-2 Vectors

The pENFRUIT collection was adapted to hpRNAi strategies with the addition of two plasmids pENTR_RNAi (pEFS4r-3r and pEFS3-2) and the construction of a new Destination vector (pDest/EFS). Both pEFS3-2 and pEFS4r-3r vectors share the same cloning region (polylinker). This region contains *SacI*, *SmaI*, and *XhoI* sites and is set in reversed orientations in each plasmid. For hpRNAi strategies, a fragment of the GOI needs to be cloned into both pEFS4r-3r and pEFS3-2 using two of the restriction enzymes of the polylinker. The GOI fragment is, in this way, inserted in inverted orientations in separate plasmids. Between the two cloning sites spans a fragment of a tomato intron which is subsequently removed by splicing. The pDest/EFS Destination vector, also incorporated in the collection, is suitable for hpRNAi strategies, as it contains a 35S terminator sequence downstream of the Gateway *attR1-attR2* recombination sites. This enables the making of different constructions for silencing strategies driven by user-defined pEF1-4 promoters (8). The types of vectors used for the expression or silencing of a GOI are summarized in Table 1.

Table 1
Types of Gateway vectors used in the three-fragment MultiSite recombination reaction for the construction of Expression vectors aimed to express or silence a gene of interest in plants

Vector	Type	Recombination sites	Application	Goal
pDONR 221 P4r-P3r	Donor vector	<i>attP1/attP4</i>	BP cloning	GOI expression
pEF1-PROM-4	Entry vector	<i>attL1/attL4</i>	LR cloning	GOI expression
pEF4r-GOI-3r	Entry vector	<i>attL4r/attL3r</i>	LR cloning	GOI expression
pEF3-TER-2	Entry vector	<i>attL3/attL2</i>	LR cloning	GOI expression
pKGW ₀	Destination vector	<i>attR1/attR2</i>	LR cloning	GOI expression
pEFS4r-GOI-3r	Entry vector	<i>attL4r/attL3r</i>	LR cloning	GOI silencing
pEFS3-IOG-2	Entry vector	<i>attL3/attL2</i>	LR cloning	GOI silencing
pDest/EFS	Destination vector	<i>attR1/attR2</i>	LR cloning	GOI silencing
pEXP PROM:GOI:TER	Expression vector	<i>attB1/attB2</i>	Expression in plant	GOI expression
pEXPhp PROM:GOI	Expression vector	<i>attB1/attB2</i>	Expression in plant	GOI silencing

PROM promoter sequence, GOI gene of interest, TER terminator sequence

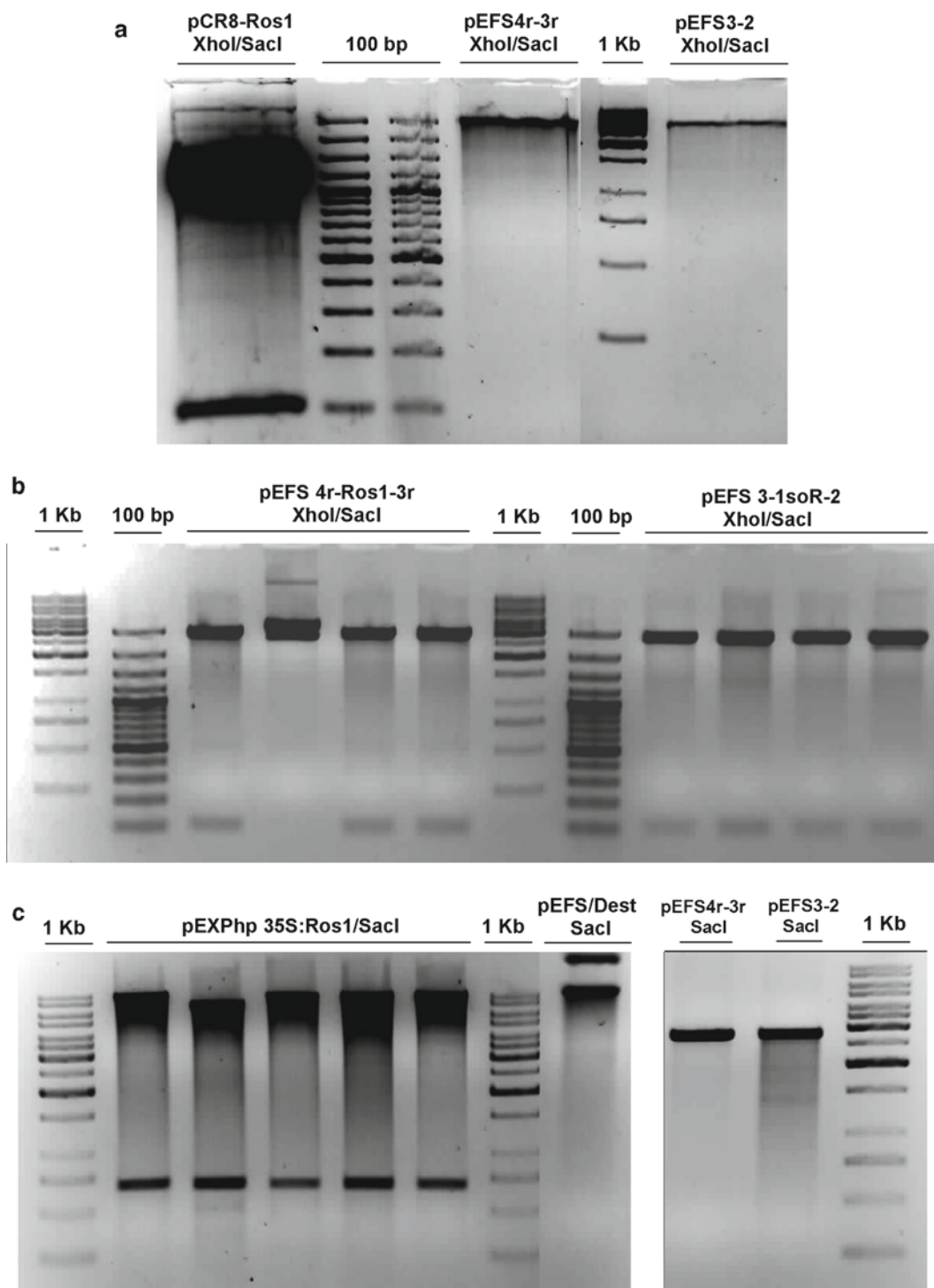


Fig. 2. Isolation of *Rosea1* 100-bp gene fragment (Ros1) and cloning into pEFS4r-3r and pEFS3-2 vectors. (a) Ros1 fragment was isolated after 3 h of double enzymatic digestion with *XhoI/SacI* at 37°C from 6.2- μ g pCR8ROS plasmid DNA. The same double digestion conditions were used to get pEFS4r-3r- and pEFS3-2-linearized plasmids, in this case from 2.04 to 3.38 μ g, respectively. (b) Ros1 100-bp fragment and the linearized pEFS vectors were gel extracted and purified. Two parallel T4 ligation reactions were performed overnight at 24°C, which required 5.94 ng (90 fmol) of purified Ros1 fragment DNA and 58 ng (30 fmol) of both linearized pEFS vectors. After *Escherichia coli* transformation, eight colonies were grown

1. Amplify a fragment of the GOI of approximately 100 bp in length using oligonucleotides containing two different restriction sites from the polylinker (*SacI*, *SmaI*, or *XhoI*) (see Note 4). PCR conditions will depend on fragment length and oligonucleotide T_m constrictions. Digest the pEFS4r-3r and pEFS3-2 vectors and the GOI fragment using the same enzymes in three different Eppendorf tubes in order to get “sticky ends.” The GOI fragment can also be obtained from a vector by double enzymatic digestion using a combination of *SacI*, *SmaI*, or *XhoI* restriction enzymes, thus avoiding the oligonucleotide design and amplification. Proper enzymatic reaction conditions can be found at the enzyme manufacturer website (see Notes 5 and 6). An example of GOI fragment isolation is shown in Fig. 2.
2. Purify GOI fragment and pEFS4r-3r- and pEFS3-2-linearized vectors using QIAquick Gel Extraction Kit (QIAGEN).
3. Prepare two 1.5-mL Eppendorf tubes and set two ligation reactions (pEFS4r-3r-GOI and pEFS3-2-GOI) using T4 DNA ligase (Invitrogen) and the conditions for cohesive ends recommended by the manufacturer (insert:vector molar ratio of 3:1 and 23–26°C), for instance, 57 ng (30 fmol) of each pEFS vector (20 ng/μL) and 6 ng (90 fmol) of 100-bp GOI fragment (19 ng/μL). After 1 h or overnight ligation reaction, dilute the ligation volume one-fifth in each tube and proceed to transform *E. coli* chemically competent cells (see Note 7).
4. Proceed as described in Subheading 3.1 step 6, pick colonies, and carry out DNA minipreps. Confirm positive clones by *SacI*, *SmaI*, and *XhoI* restriction analysis. An example of gel confirmation is shown in Fig. 2. Sequencing with M13 oligonucleotides may be required in order to confirm if the GOI has been cloned into pEFS4r-3r and pEFS3-2 vectors.

**3.3. Three-Fragment
MultiSite Gateway
LR Recombination
Reactions: Generation
of pEXP_PR:GOI:T
or pEXPh P:GOI
Binary Plasmids
Ready for
Agrobacterium
Transformation**

1. Prepare vectors required for the triple LR recombination reaction. For instance, in case of *GFP* expression driven by NH promoter, pEF1-NH-4, pEF4r-GFP-3r, and pEF3-Tnos-2 Entry vectors and pKGW,0 Destination vector would be used for the construction of pEXP NH:GFP:Tnos Expression vector. In another example, the gene silencing of *Rosea1* driven by the CaMV35S promoter would require pEF1-35S-4, pEFS4r-Ros1-3r, and pEFS3-1soR-2 Entry vectors and pDest/EFS Destination vector in order to make the pEXPhp 35S:Ros1 Expression vector.

Fig. 2. (continued) (four for each ligation), and 20 μL of each plasmid miniprep was double digested with *XhoI/SacI* at 37°C for 1 h. Enzymatic digestions were checked on a 1.5% agarose TAE 1× electrophoresis gel which revealed the 100-bp Ros1 fragment in seven of eight digestions. (c) After an LR recombination reaction using pEF1-CaMV35S-4, pEFS4r-Ros1-3r, pEFS3-1soR-2, and pEFS/Dest vectors, the pEXPhp 35S:Ros1 Expression vector was obtained. As a result of *SacI* digestion, two fragments were produced: one of 9,757 bp and another of 709 bp, this latter corresponding to the tomato intron flanked by 100-bp *Rosea1* gene sequences. *SacI* did not cut pEFS/Dest and linearized pEFS4r-3r and pEFS 3-2 vectors.

2. For each LR reaction, use a 1.5-mL sterile Eppendorf empty tube. In every tube, add 10 fmol of each Entry vector, 1 μ L of the corresponding Destination vector (20 fmol), and TE buffer to reach a total volume of 8 μ L. Then, add 2 μ L of LR Clonase II to each LR reaction tube, mix by vortexing, and incubate at 25°C for 16 h using a thermoblock (Thermolyne Dri-Bath Model 17600). To stop the reaction, add 1 μ L of Proteinase K solution and incubate the tubes at 37°C for 10 min.
3. Transform chemically competent *E. coli* cells, One Shot Mach1 T1R competent cells (Invitrogen) using 2 μ L of LR reaction following manufacturer's instructions (see Note 8). Plate cells in 50- μ g/mL spectinomycin LB agar plates. Put the plates in a 37°C incubator for 16 h.
4. Proceed as described in Subheading 3.1 step 6 by picking colonies and carrying out DNA minipreps. Analyze the DNA by restriction using the proper enzymes. For instance, a digestion with enzymes *Sac*I, *Sma*I, or *Xho*I is appropriate in the case of Expression vectors for RNAi as it allows the isolation of the fragment corresponding to the hairpin intron flanked by both GOI sequences. As an example, the construction of pEXPhp 35S:Ros1 vector aimed at silencing the *Rosea1* gene in transgenic tomato and tobacco plants is described in Fig. 2. Additionally, the sequencing of the Expression vector can contribute to check if constructions have been correctly assembled. Similarly to the BP reaction (see Note 2), the length of the fragments to recombine has a negative impact on the performance of the LR reaction (see Note 9).

3.4. *Agrobacterium tumefaciens* C58 Transformation

1. Add 1 μ L from a positive *E. coli* plasmid miniprep of each construction to a sterile microcentrifuge tube containing 40 μ L of electrocompetent cells of C58 *Agrobacterium tumefaciens* and electroporate samples using an electroporator (Bio-Rad, gene-pulser model 165-2077) (see Note 10). Add 250 μ L of SOC medium maintained at room temperature and incubate in a 15-mL plastic cap tube at 28°C for 2 h with agitation. Then, spread 50–150 μ L of the bacteria suspension on solid LB plates containing 50 μ g/mL spectinomycin and 50 μ g/mL rifampicin antibiotics. Incubate for at least 48 h at 28°C.
2. Check if the transformation has worked for every construction by picking 5–10 colonies from each culture plate (in sterile conditions) and incubate them in liquid LB culture containing spectinomycin and rifampicin (50 μ g/mL each) antibiotics at 28°C for at least 48 h. Again, carry out DNA minipreps and digestions with the appropriate restriction enzymes to confirm the constructs (see Note 11).

3.5. Agroinjection of Tomato Fruits for Transient Expression Assay: Analysis of Promoter Activity as an Example

Transient expression of genes can be used prior to stable transformation of plants to produce a large amount of protein or verify expression constructs (14). A frequently used technique is agroinfiltration on plant leaves (13), and we have used this technique to evaluate the functionality of some constructs as in the case of *Rosea1* RNAi Expression vector (see Subheading 3.6). As we are more interested in tomato fruit biotechnology, our laboratory has developed a method based on leaf agroinfiltration that was adapted to transient expression of foreign genes directly into the tissues of the fruit. The method involves the injection of a suspension of *Agrobacterium* cells into the fruit by using a syringe needle. The injection is made through the stylar apex of the fruit so that the infiltrated solution reaches the entire surface of fruit (11). We performed transient expression assays using *GFP* as reporter. The aim was to compare the activity of newly isolated fruit-specific promoters (for instance, PNH), against ripening-related fruit promoters (such as E8) and against a constitutive promoter (CaMV35) in two different stages of fruit ripening: mature green and breaker. These promoter sequences were cloned next to a GFP/GUS translational fusion and tested in transient expression assays (8). For the quantification of GFP fluorescence levels, though, the introduction of an additional fluorescent gene as internal standard-like DsRed was necessary. For this purpose, the tomato fruits were coinjected with another *Agrobacterium* strain containing a CaMV35S:DsRed construct, and tomato slices were simultaneously double-scanned to determine signal ratios between green and red fluorescence channels.

1. For every construction, pick an *Agrobacterium* colony into a 50-mL culture tube containing 3 mL of YEB medium, 2 mM MgSO₄, and spectinomycin and rifampicin (50 µg/mL each) antibiotics. Grow the cultures at 28°C with shaking (150–200 rpm) for 36–48 h inside an incubator.
2. Prepare a subculture by transferring 50 µL of the previous culture into a 50-mL tube containing 3 mL of YEB medium, 2 mM MgSO₄, and spectinomycin and rifampicin (50 µg/mL each) antibiotics. Grow at 28°C with shaking (150–200 rpm) for 16–24 h.
3. Collect the *Agrobacterium* culture by centrifugation at 6,000 × *g* for 15 min using a centrifuge (Sigma-302) and resuspend it with infiltration MES buffer. Check the optical density (OD) and dilute if necessary, adding more infiltration buffer to reach 0.05 OD (see Note 12). The OD is measured at 600-nm wavelength by means of a spectrophotometer (UV/VIS Spectrophotometer SP8001, Dinko). Put cultures to incubate at room temperature in gentle agitation (20 rpm) for at least

2 h. Collect GFP/GUS-containing cultures and combine 1:9 (vol:vol) with an *Agrobacterium* C58 strain containing CaMV35S:DsRed construct (culture OD₆₀₀ = 0.05). Expression assays can be improved by adding an *Agrobacterium* strain expressing the p19 silencing suppressor (see Note 13).

4. For the infiltration process, use a 1-mL syringe with needle (25 GA 5/8 IN, needle: 0.5 mm × 16 mm, BD Plastipak™) for each mixed solution (construction/35S:DsRed). Perform infiltration by inserting the needle about 3–4 mm within the tissue of tomato fruit (*Solanum lycopersicum* cv. Micro Tom) stylar apex and by injecting the solution carefully. The infiltrated solution can be visible as it spreads throughout the inner fruit tissues, causing a darkening of the fruit. The agroinjection should finish when the fruit is fully infiltrated; at this stage, a leakage of few drops is usually observed at the tip of the sepals. The amount of injected solution varies depending on the fruit size, but 700 µL should be enough to infiltrate a whole full-grown Micro Tom fruit. Expression or silencing of the GOI can be observed 4–6 days after infiltration. Usually, the fruits are agroinjected after 25 days of the flower anthesis (25 days postanthesis or 25 dpa) in the green phase. For studies of gene expression during fruit ripening, the fruit is agroinjected at a late mature green stage (30–35 dpa) and harvested at the breaker/red stage.
5. To determine the GFP/DsRed signal ratio, a Typhoon Trio Variable Mode Imager (Amersham Biosciences) fluorescence scanner for large gels is required. Place the samples onto a low-fluorescent glass plate which has been previously cleaned with a 5% DECON90 solution and 70% isopropanol, rinsed with distilled water, and dried thoroughly using a lint-free cloth (see Note 14).
6. Harvest six tomatoes for each construction between 4 and 6 days after infiltration and cut them using a meat cutter (CF-172 Fagor) to get 1-mm-thick cross sections. Place the tomato slices (one or two for tomato) in a column distribution on the glass plate so that each column corresponds to each construction and leave two columns for 35S:DsRed and p19 control tomato cutlets. Put the glass into the Typhoon scanner and set the area and orientation using the Typhoon Scanner Control software. Use 532 nm (excitation) and 610 nm (emission) wavelength filters to detect DsRed in one channel, and 488 nm (excitation) and 520 nm (emission) filters to detect GFP in a second channel. Prescan the glass plate first at low resolution (pixel size = 500 µm) to make sure the experiment was successful and check the PMT voltage parameters to make sure the data is not saturated. Then scan the glass plate at higher resolution (pixel size = 50 or 25 µm) and save the images.

7. Run the ImageQuant Tools software (Amersham Biosciences) to open and analyze the images. For each tomato slice, modify a grid to select four circular areas with radii of 10 pixels each, located in the area of the placenta and/or gel showing DsRed fluorescence (see Note 15). Determine the ratios of fluorescence intensity of green (GFP) and red (DsRed) channels and put them in a table. Using an Excel spreadsheet, represent the ratio values by subtracting the background noise, calculated as the average of the green/red ratios in those cutlets from tomatoes solely agroinjected with 35S:DsRed. Additionally, to obtain more information on the expression patterns, transfer the constructs to *Agrobacterium tumefaciens* LBA4404 strain by electroporation and use for tomato stable transformation (15). An example of double-scanned tomato slices and green/red ratios representation of CaMV35S, PNH, and E8 promoter activities is shown in Fig. 3. The GFP expression driven by 35S

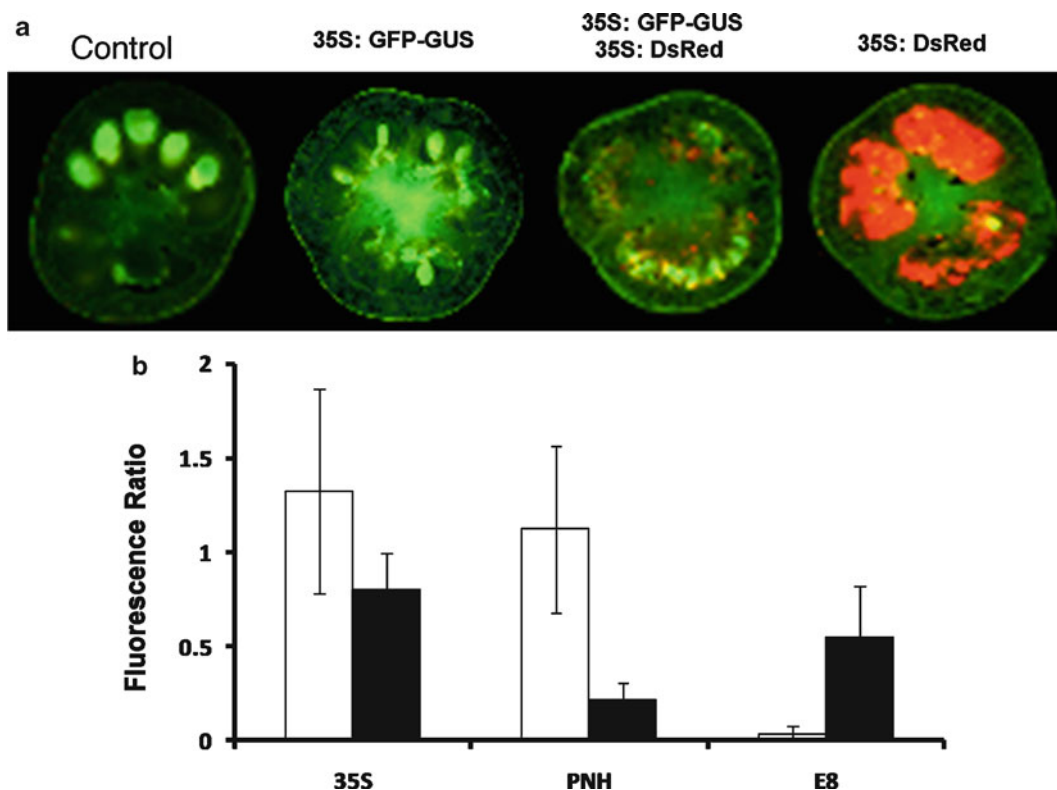


Fig. 3. Promoter analysis using transient expression. (a) Examples of scanning images obtained from sliced tomatoes transiently transformed with promoter:reporter constructs. Control tomatoes were agroinjected with a nonfluorescent construct. 35S:DsRed is used as an internal standard. (b) Green/red fluorescence ratios from promoters CaMV35S, PNH, and E8 were measured at mature green (empty columns) or breaker (filled columns) stages. For each tomato slice, four circular areas corresponding to placenta and/or gel areas showing DsRed fluorescence were selected. Background values (from CaMV35S:DsRed injected only) were subtracted from the final values. Bars represent average ratios $\pm$ standard deviation.

promoter in the green phase was the highest in the study of promoter activity in tomato fruit. In the breaker phase, its activity decreased and was comparable to that of fruit-specific promoters. PNH promoter activity decreased during ripening, contrary to what was observed with the E8 promoter whose activity increased during the fruit ripening, as described in the literature (16).

**3.6. Checking
Functionality of
hpRNAi Constructs:
Transient Silencing
of *Rosea1* Transgene
as an Example**

1. For transient hpRNAi experiments, syringe-infiltrate transgenic *Ros1* tobacco leaves constitutively expressing the *Antirrhinum majus Rosea1* gene with an *Agrobacterium* culture (OD = 0.05) carrying pEXhp 35S:*Ros1*, following a standard leaf agroinfiltration protocol, as described in ref. 13. *Ros1* plants show a bronze-coloured phenotype as a result of the activation of flavonoid biosynthesis promoted by the *Rosea1* transcription factor (Butelli E and Martin C, unpublished). The successful silencing of *Rosea1* transgene in an hpRNAi strategy is easily detectable as a reversion to the green wild-type phenotype. Three weeks later, the phenotype of the infiltrated leaves was scored as represented in Fig. 4a.

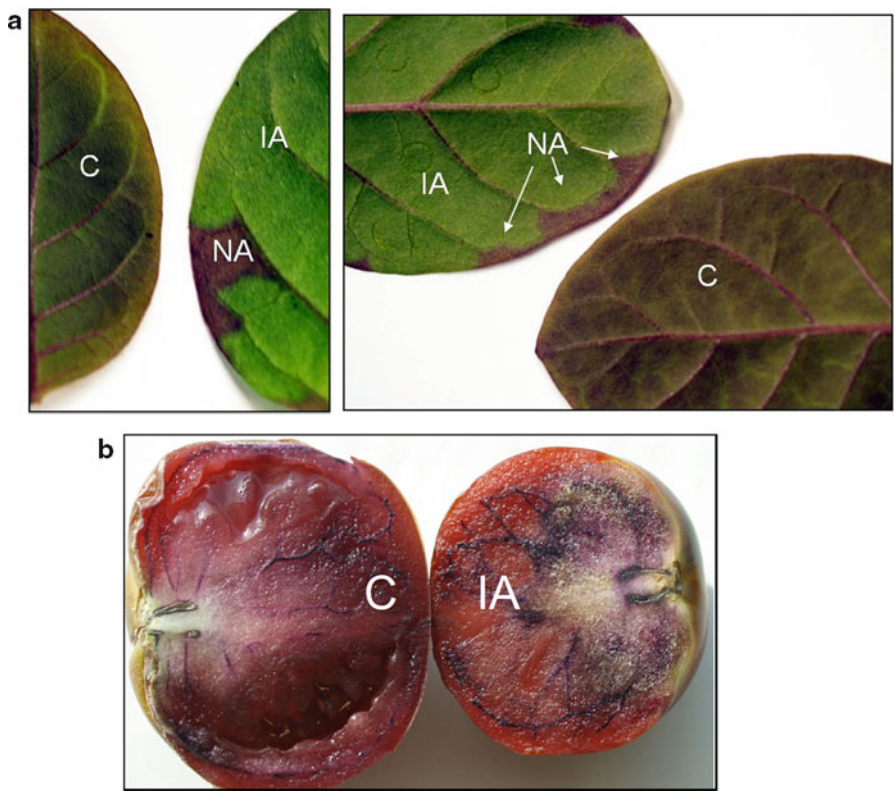


Fig. 4. *Rosea1* gene transient hpRNAi silencing in 35S:*Rosea1* (*Ros1*) tobacco leaves (a) and E8:*Rosea1*-E8:Delila tomato fruits (b). IA infiltrated areas of the organ, NA noninfiltrated areas of the same organ (only applicable to leaves, where infiltration zone can be visually monitored at the time of infiltration), C control, noninfiltrated organ.

2. A similar strategy was followed using an anthocyanin-rich transgenic tomato line derived from F6DR line (17) carrying *Rosea1* and *Delila* transgenes (18). Agroinject an *Agrobacterium* culture (OD=0.05) carrying pEXhp 35S:Ros1 into mature green fruits, as described in the previous section. Here, agroinjection of pEXhp 35S:Ros1 construct should result in a partial reversion from light-purple to the red (wild-type) tomato colour. The result of the silencing experiments was recorded 3 weeks after agroinjection and is shown in Fig. 4b.

4. Notes

1. Because of their length, we recommend ordering highly purified *attB4r/attB3r* oligonucleotides (HPLC or PAGE purification). We got recombination problems or obtained no PCR product at all when using desalted only oligonucleotides.
2. We have confirmed that high frequency of A/T nucleotides (in promoter sequences for instance) affects BP reaction yield. We have followed PEG-based purification protocol (as suggested by Invitrogen to remove *attB* primer dimers), as a substitute of gel purification in some cases of unsuccessful BP reaction, and we obtained good results. Usually a 30% PEG/Mg solution is supplied in BP Clonase II Kits.
3. Invitrogen suggests BP incubations longer than 1 h for large PCR products (≥ 5 kb). We have confirmed an increase in the yield of colonies after overnight incubations; however, a good yield can be obtained with incubations of 1 or 2 h for 1–2 kb PCR products.
4. Posttranscriptional gene silencing (PTGS) is produced by small RNAs and has been proved that fragments of the GOI of approximately 100 bp are enough to trigger an efficient PTGS response (19, 20).
5. An hour of enzymatic digestion may be enough, but these restriction enzymes are suitable for an overnight digestion in case of poor band yielding.
6. To ensure the linearization of pEFS vectors, we suggest an overnight enzymatic digestion. A treatment of 30 min (37°C) of vector molecules with calf-intestinal alkaline phosphatase (Promega Corporation, Madison, USA) to remove the 5'-phosphate groups can be used to prevent recircularization.
7. Although the manufacturer suggests that 1 h of ligation reaction is sufficient in case of cohesive ends, we have obtained better results with an overnight reaction at 24°C. We have also found that one-fifth dilution of the ligation reaction is more efficient in *E. coli* transformation, as suggested by the manufacturer.

8. You can use one type or another of *E. coli* competent cells; both showed similar transformation efficiencies. However, the Mach1 T1R cells are recommended by Invitrogen for their faster growing rate.
9. We have checked that overnight LR reactions increase the number of colonies containing the desired Expression clone. Also for large (>10 kb) Entry clones or Destination vectors, linearizing the Entry clone or Destination vector increases the LR efficiency, as pointed by Invitrogen.
10. In tomato, *Agrobacterium* C58 strain is more efficient in transient expression assays, while other strains like LBA 4404, for instance, are more suitable for plant stable transformation.
11. To improve *Agrobacterium* plasmid miniprep yield of low-copy-number plasmids, pellet the cells from 10 mL liquid LB medium and follow the QIAprep Spin Miniprep Kit (QIAGEN) procedure. Plasmid yield improves by adding 20 μ L of phosphatase alkaline (CIP) (New England Biolabs) to the column and incubating the column in ice for 15 min prior to the miniprep's washing step.
12. Experimental assays performed at different ODs have proved that OD₆₀₀ values of 1.0–0.5 resulted in low reporter gene (for instance, *GFP*) expression levels. We have concluded that a more diluted bacteria culture (0.05–0.005 OD₆₀₀ values) results in a better distribution of GFP fluorescence in the fruit.
13. PTGS can be reduced by coexpression with a suppressor of viral proteins, such as p19 protein (21). In our lab, tomato fruits have shown higher expression values and longer time stability of the protein of interest, when GFP/GUS expressing cultures were coinjected 1:20 (vol:vol) with pBIN 61 P19 (OD₆₀₀ = 0.05) *Agrobacterium* p19-expressing strain.
14. Dust fluoresces and scatters light, which causes artifacts on images and can interfere with quantification. To avoid this, wear powder-free gloves and use clean and rinsed low-fluorescent glass plates.
15. The highest accumulation of GFP protein takes place in the placental tissue which is more accessible to the bacteria. In some cases, fluorescence can be observed in the inner surface of the pericarp; however, this tissue remains impregnable and should be studied by other techniques.

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A One-Time Inducible Transposon to Create Knockout Mutants in Rice

Yuh-Chyang Charng

Abstract

Use of a transposon is an efficient tagging tool for exploring the function of the gene it inserts into or is adjacent to. A few modifications have been applied to the native *Ac* transposon to allow it to transpose efficiently or spontaneously and stop quickly thereafter. Furthermore, locating the transposon between a constitutive plant promoter and a reporter gene, such as the firefly luciferase gene, allows for nondestructively detecting excision events *in vivo*. This chapter describes a detailed protocol for one-time inducible transposon tagging of rice cells and their subsequent screening and regeneration into mutant lines.

Key words: *Ac* transposase, Inducible promoter, Knockout (or on) mutant, Salicylic acid, Luciferase gene

1. Introduction

To date, many plant mutants have been obtained by classical approaches such as γ -rays or EMS mutagenesis (1–3). Although generating mutants by these treatments is relatively easy, subsequent identification and isolation of the implicated genes requires time-consuming cloning by functional complementation (3). Insertional mutagenesis with transposons (4, 5) or T-DNA vectors (6–8) has been used successfully to isolate plant genes identified by the phenotype of their mutant alleles. This technique, termed gene tagging, has been used extensively for many plant species. By using T-DNA vectors, genetic transformation methods have been established to efficiently transfer T-DNA from *Agrobacterium tumefaciens* into plant genomes. However, use of transposons is a good tool for tagging experiments for plants species with low *Agrobacterium*-transformation efficiency or requiring saturated mutagenesis. Yet a major difficulty in use of native transposon as the tagging tool is to

trigger efficient transpositions and limit subsequent transposition events (for yielding stable mutants). Understanding the behavior of transposons in host species and sometimes modifying transposons for stable mutants have led to their use in heterologous plant species with high efficiency (9, 10).

Previously, this author fused the PR-1a promoter with the *Ac* transposase gene to develop inducible transposon systems for plants such as rice and tobacco (11). As well, a one-time transposon system was created by locating one end of the transposon in the intron of the *Ac* transposase gene to yield stable mutants (12). Treatment with salicylic acid (SA) induced transposition of this transposon, *COYA*, and led to transposase gene breakage in exons. The modified transposon was constructed between a constitutive promoter and the firefly luciferase (LUC) gene. Transposition events could be nondestructively detected *in vivo* according to the restored activity of the LUC reporter gene. Then, using TAIL-PCR or inverse PCR (13, 14) allows for amplifying genomic sequences flanking transposon insertions. This chapter describes how to induce transposition events efficiently to create rice mutants and how to screen and enrich the illuminant mutants. Expanding the usage of the one-time inducible transposon, that is, further modifying *COYA* for carrying constitutive enhancer elements that can cause transcriptional activation of flanking plant genes, will increase the scope of the screening to knockout as well as knockon mutations (15).

2. Materials

1. Transgenic rice calli: A one-time inducible transposon, *COYA* (Fig. 1), was transformed into wild-type rice (*Oryza sativa* L. cv. TNG67) (16). Successful transgenic rice lines were analyzed by DNA blot and PCR (e.g., TAIL PCR) to determine the transgene copy number and location. Transgenic start lines containing one copy of the untransposed inducible transposon (see Note 1) were determined and cropped to set seeds.
2. Callus induction and regeneration medium (CIM): 3.98 g/L N6 salts and vitamins (17), 30 g/L sucrose, 0.3 g/L casamino acids, 2.8 g/L proline, 2 mg/L 2,4-D, and 2 g/L Gelrite, pH 5.7.
3. Inducer: 1 mM SA (sodium salt, Sigma, USA).
4. Luciferin solution: 0.4 mM d-luciferin (potassium salt, Synchem, Germany) in water, sterilized by 0.45- μ m membrane filter (Millipore, USA).
5. Shoot and root regeneration medium (RM): 3.98 g/L N6 salts and vitamins (17), 30 g/L sucrose, 0.3 g/L casamino acids, 2.8 g/L proline, 2 mg/L 2,4-D, and 2 g/L Gelrite, pH 5.7.

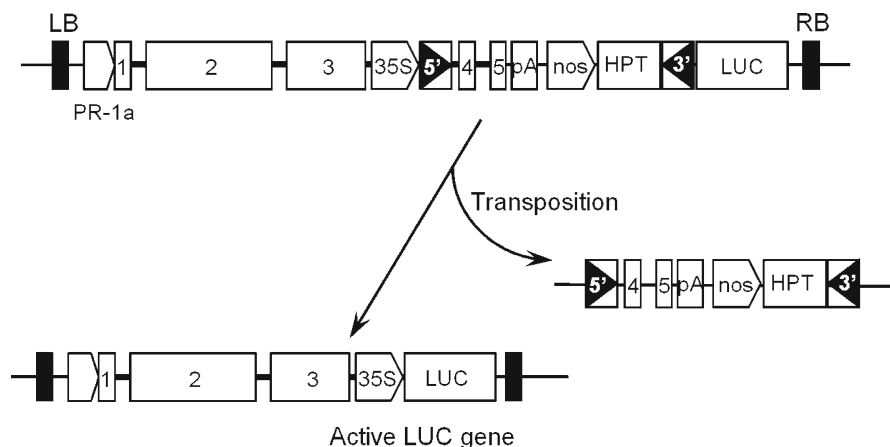


Fig. 1. Schematic diagram of the one-time transposon system *COYA*. *LB* left border, *RB* right border, *5'* and *3'* *Ac* left and right terminal-inverted repeat, *PR-1a* PR-1a inducible promoter, *HPT* hygromycin phosphotransferase gene, *pA* poly(A) fragment, *nos*, nopaline synthase promoter, *LUC* luciferase gene, *1–5* transposase gene exon 1 to exon 5 (modified from ref. 12).

3. Methods

The one-time inducible transposon system for rice gene tagging is based on several features: (1) high regeneration efficiency of rice calli, (2) high transposition efficiency of *Ac*-based transposons in rice calli, (3) chemically induced promoter fusion, with the transposase resulting in the “controlled” transposon, and (4) use of the LUC reporter gene as a nondestructive method to screen rice mutants. The construct containing the inducible transposon was introduced into rice plants according to the standard procedure (16). In general, transgenic lines containing one T-DNA insert were collected to assess the induced transposition efficiency and to create or screen mutants with the desired rice lines. Seeds of transgenic start lines containing a hemizygous transgene (see Note 2), which would be subsequently determined by PCR-based genotyping with embryo-derived calli, were used to create knockout mutants as follows.

3.1. Seed Sterilization and Calli Proliferation

1. Sterilize dehulled seeds with 2% NaOCl + 2–3 drops of Tween 20 for 15 min.
2. Wash seeds with sterilized ddH₂O for at least five times.
3. Inoculate seeds on CIM (five seeds per 90-mm plate) and incubate at 28°C in constant light for 3–4 weeks.
4. Extract DNA of radicle tissue from each embryo and perform PCR genotyping to collect the hemizygous transgenic rice lines.
5. Perform preliminary luminant assay (see Subheading 3.3) of each plate containing the embryo-derived calli. Any luminant sector from calli indicates spontaneous transposition events (see Note 3).

In that case, incubate the illuminant rice calli incubated on CIM containing hygromycin for 2–4 weeks (see Subheading 3.3 step 3c). Collect the luminant-negative calli for SA induction.

3.2. Induction by SA

1. Transfer rice calli to CIM containing 1 mM SA and incubate for 1 week (see Note 4).
2. Transfer rice calli to normal CIM for calli regeneration on CIM and then incubate for 4–8 weeks (see Note 5).

3.3. Detection and Enrichment of Rice Calli Containing Transposition Events—In Vivo LUC Assay

1. Nondestructive detection of transposition events in transgenic rice calli requires the excision of transposon to relink the luciferase gene to a plant promoter. An example would be the construct shown in Fig. 1, in which excision of the *COYA* transposon restores the luciferase activity based on the CaMV 35 S RNA promoter-luciferase fusion (12).
2. To visualize the luciferase reaction in rice calli, apply 5 mL sterilized luciferin solution on 90-mm plate material containing CIM for at least 10 min, depending on the size of the rice calli. Remove the solution before capturing images (see Note 6).
3. To detect light with video equipment, this author uses sophisticated instrumentation composed of a dark chamber containing a 16-bit CCD camera, with zoom lens of 11.5–69 mm to penetrate the illuminants (KURABO Chemi-Stage CC16, Osaka, Japan). This equipment is linked to a PC and an image analysis system (UniTek Dolphin 1D, Taipei, Taiwan). The luminescence image can be captured with the desired exposure filming process (see Note 7).
 - (a) Position the sample in the dark chamber and set the reflected light reference image with epi white light (see Note 8).
 - (b) Create luminescence images in the dark after collecting light during 5–20 min with incremental filming. The amount of exposure time for one filming is the exposure time divided by the number of partitions. The process overlays the images according to the number of partitions. Typically, a 20-min exposure with four partitions yields distinct images.
 - (c) Map the luminescence image with the reference image to collect illuminant rice calli (Fig. 2). Incubate the illuminant rice calli on CIM containing hygromycin for 2–4 week (see Note 9). Repeat step 3 until each illuminant rice callus regenerates as an independent mutant line (see Note 10).

3.4. Screening of Rice Insertional Mutants

The transposon has been used for studying rice gene function with both forward and reverse genetics approaches. The latter approach predominates in some labs because of limited space. Forward genetics is the traditional approach to genetics, which starts with a phenotype and then identifies the gene(s) or mutation that control

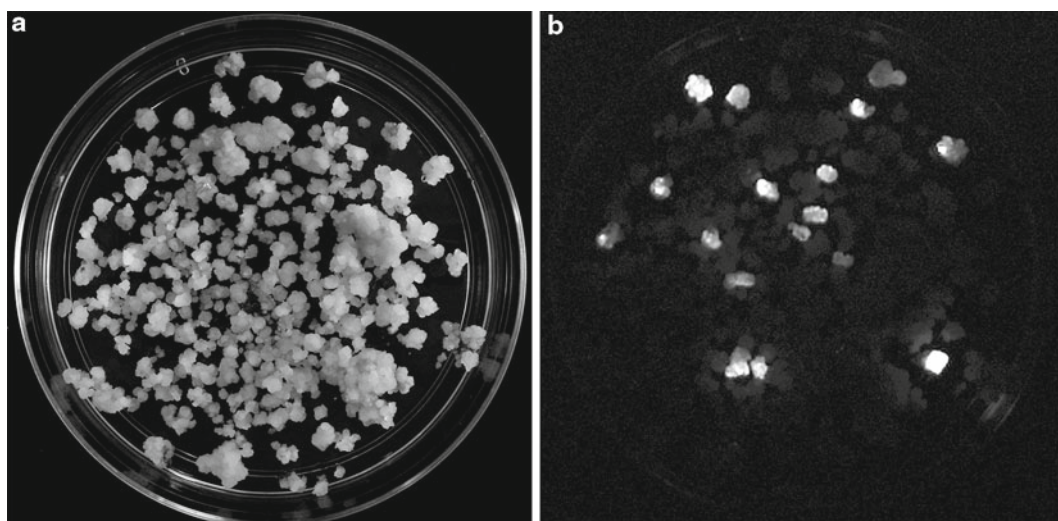


Fig. 2. Screening the illuminant rice calli, which contain transposition events after salicylic acid induction. (a) Reflected-light image of transgenic rice calli harboring *COYA* system and (b) its luminescent image.

or cause that trait. This process contrasts with reverse genetics, whereby a gene or sequence is used to determine the phenotype. Both genetic screenings of insertional mutants have been used in detail in *Arabidopsis* (18, 19). This section briefly describes the main differences according to the start materials and the transposon construct. Because the excision of the *COYA* transposon restores the LUC activity, determining the empty donor sites is not needed (19). Furthermore, all illuminant calli are incubated on media containing hygromycin, which ensures the reintegration of *COYA*. Southern blot analysis does not need to be performed to determine the complete (cut-and-paste) transposition events.

3.4.1. Forward Genetics Approach

- a. Transfer the illuminant calli to RM for shoot and root regeneration. Grow the individual seedlings in the greenhouse and allow them to self-pollinate. Identify mutant lines with a phenotype of interest (see, e.g., refs. 20–22) (see Note 11).
- b. Prepare DNA of rice leaves.
- c. Amplify the flanking sequences by TAIL PCR (13).
- d. Purify the PCR product and sequence it.
- e. Search databases to identify the insertion site.

3.4.2. Reverse Genetics Approach

- a. Prepare DNA of illuminant rice calli.
- b. PCR-based screening of genomic DNA from mutant lines by use of a combination of tag- and gene-specific primers (19).
- c. Identify homozygous mutant plants.

4. Notes

1. In general, a start rice line for creating knockout mutants is determined according to the inducer sensitivity of the transgene it contains (i.e., it yields no [or rare] spontaneous transposition events but high transposition efficiency by applying the inducer). The start lines are maintained by cropping individual rice plants in pods and collecting the LUC-negative progenies.
2. Transgenic lines containing homozygous transgene (2 *COYA* copies) can also be used as start lines to induce transposition. However, the reintegration of the excised *COYA* must be confirmed (e.g., by TAIL PCR) after obtaining the illuminant rice calli. The untransposed copy shows that hygromycin-resistant activity can rescue the pseudomutant lines containing the partially transposed copy.
3. Spontaneous transposition events might be triggered by endogenous stimuli in rice plants. Although this happens rarely (in this author's lab), these events may sometimes be transmitted to the progenies. In that case, the whole embryo (and derived calli) will yield a luminoimage. This embryo can be expected to be a new mutant line and used to perform forward or reverse genetic studies. If many sibling embryos are LUC positive, they might have resulted from the same transposition event and therefore yield only one mutant line. In that case, this transgenic rice line should be excluded as a start line for creating abundant SA-induced mutants.
4. The transposition efficiency of PR-1a::TPase-based *Ac* transposon is dose dependent (11). Yet a high SA concentration inhibits the regeneration of rice calli. Furthermore, the optimal SA concentration to trigger the highest transposition efficiency is line dependent. In general, most PR-1a::TPase-based *Ac* transposons yield good efficiency when treated with 1 mM SA for 1 week.
5. As expected, *Ac*/*Ds* excision events are triggered predominantly in cells actively undergoing cell division (23, 24). Therefore, further regeneration of rice calli is important for transposition. Removing the SA inducer during the calli regeneration avoids the possibility of the inverse dosage effect, that is, a high level of transposase expression sometimes inhibits transposition (25). Removal of the SA inducer can stop triggering the transposase expression and, furthermore, yield the capacity of "optimal" transposase dosage for transposition because of the

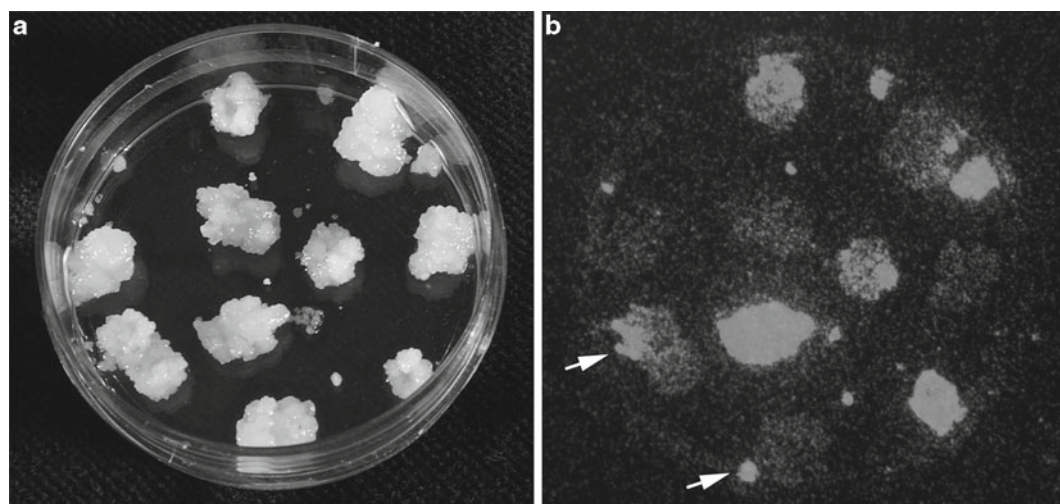


Fig. 3. An example of chimeric illuminant rice calli after preliminary screening. (a) Reflected-light image of regenerated rice calli (photographed with regular digital camera). (b) Rice luminescence image. Arrows indicate the sectors yielding luminescence (from ref. 12).

natural protein degradation pathway. Therefore, transposition events are expected to occur frequently during rice calli regeneration after removal of the SA inducer.

6. Excess luciferin solution may yield pseudosignals because of reflection. The luciferin solution can be reused.
7. Because the desired rice calli (illuminant) should be reincubated on CIM for regeneration, all rice calli should be imaged in a Petri dish with a lid to retain sterilization.
8. Alternatively, the reference image can be obtained with a regular digital camera before applying luciferin.
9. This incubation process will ensure the reintegration of *COYA*.
10. Because all calli are arranged close together for preliminary screening, sometimes a few LUC-negative calli are chosen for further regeneration. These transgenic rice lines can be easily excluded by subsequent luminoassays. Another observation shown in Fig. 3 indicates the existence of a chimeric illuminant (only a sector of a single callus yields a light image). In that case, the illuminated portion from rice callus should be collected for another roll of enrichment until the desired line is formed.
11. Transposons containing an enhancer for activation tagging would allow for easier performance of forward genetics.

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Marker-Free Gene Targeting by Recombinase-Mediated Cassette Exchange

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and Atsushi Komamine

Abstract

Repeated gene targeting by recombinase-mediated cassette exchange (RMCE) is an efficient tool for the study of gene function and regulation because of the high predictability and repeatability of gene expression. We have developed the site-directed integration (SDI) vector system for *Agrobacterium*-mediated transformation to precisely integrate a single copy of a desired gene into a predefined chromosomal locus in the absence of any coexpressed selection marker gene (Nanto et al. Plant Biotechnol J 3:203–214, 2005; Nanto and Ebinuma Transgenic Res 17:337–344, 2008; Nanto et al. Plant Cell Rep 28:777–785, 2009; Ebinuma and Nanto (2009) Marker-free targeted transformation, in *Molecular techniques in crop improvement* (2nd Edition). (Jain, S. M. and Brar, D. S. eds.), Springer Netherlands, pp. 527–543; Ebinuma and Nanto in preparation). The SDI vector system consists of a target vector to introduce the target cassette and an exchange vector to reintroduce the exchange cassette for gene replacement. We describe the molecular design and experimental protocol that can efficiently enrich RMCE events through the removal of randomly integrated copies and select clean marker-free targeted transgenic plants by using a negative marker.

Key words: Site-specific recombination, RMCE, Targeted transformation, Marker-free, Negative marker, *CodA* gene

1. Introduction

In plants, *Agrobacterium*-mediated methods are widely used for transformation because the complete desired gene is generally assumed to be integrated with a lower copy number than direct gene transfer methods. However, even when the same gene is introduced into the same plant materials under the same culture conditions, transgenic plants still show large variations in transgene expression levels because of a lack of control of copy number and site specificity in integration. Therefore, cumbersome screening

procedures are required to identify transgenic plants with a single copy of transgenes at appropriate expression levels. Repeated gene targeting is expected to be the most promising approach to solve this problem. Recently, with the aid of a site-specific recombination system, RMCE methods to replace a desired gene with a target gene at a predefined chromosomal locus are being developed (1–7). *Agrobacterium* introduces a small amount of linear single-stranded DNA, which is not a suitable substrate for recombinase, into the plant nucleus. We reported the development of the SDI vector system for producing targeted transgenic plants and demonstrated that *Agrobacterium*-mediated methods are useful for targeted transformation by RMCE (1–5). This chapter presents the molecular design (Fig. 1) and experimental protocol of the SDI vector system for integration of a marker-free transgene into a predefined chromosomal locus.

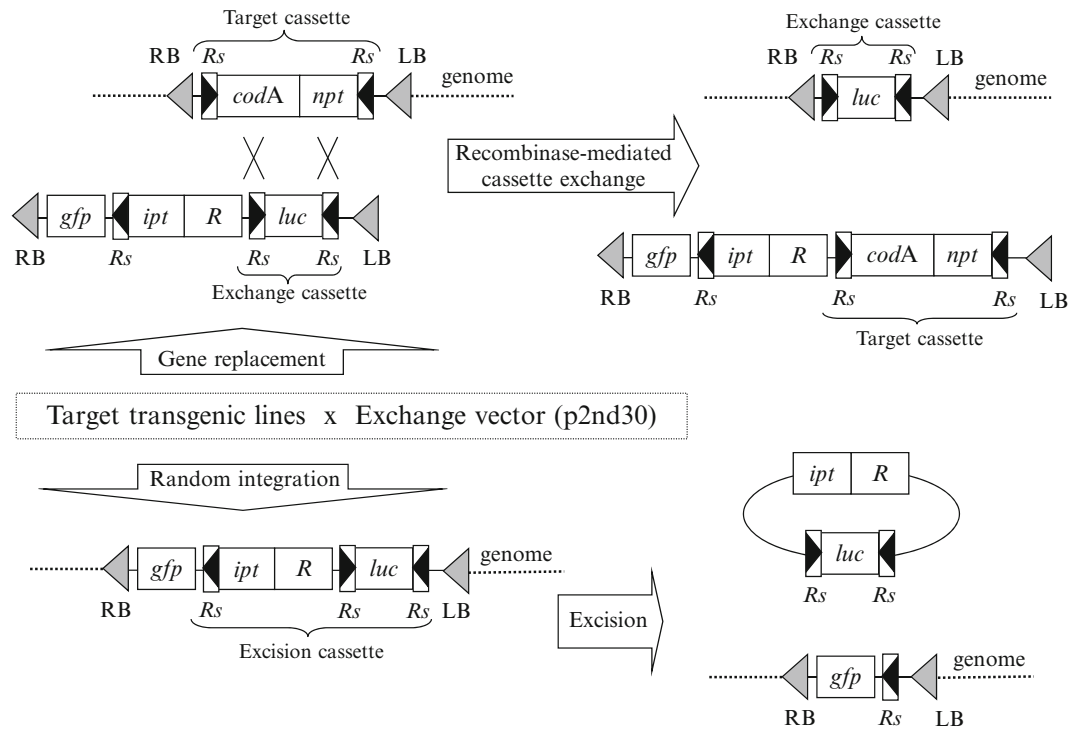


Fig. 1. Experimental design of the SDI system for RMCE. A single copy of the target cassette (*npt* and *codA* genes) flanked by two oppositely oriented *R_s* sites is located on a chromosome. The exchange cassette (*luc* gene) of an exchange vector is flanked by two oppositely oriented *R_s* sites. When an exchange vector is introduced, recombinases catalyze double crossover between the two *R_s* sites to replace the target cassette with the exchange cassette. The *ipt* and *R* genes and the exchange cassette of an exchange vector are flanked by two directly oriented *R_s* sites (excision cassette). When an exchange vector is integrated into a random locus on a chromosome, recombinases remove it. Transgenic plants with only an exchanged copy are identified as normally rooted plants on the MS agar medium containing 5-fluorocytosine (5-FC) because they lose the *codA* and *ipt* genes. *codA* cytosine deaminase gene, *npt* neomycin phosphotransferase gene, *gfp* green-fluorescent protein gene, *ipt* isopentenyl transferase gene, *R* recombinase gene, *luc* firefly luciferase gene, *R_s* recombination site, *RB* and *LB* right and left border sequences of a T-DNA.

2. Materials

1. SDI target vector plasmids: The pTSspsRScodN vector plasmid, a derivative of pBI121, has a target cassette containing *npt* and *codA* genes flanked by oppositely oriented recognition sites (*R_s*) between left and right border sequences (Figs. 1 and 2) (5).
2. SDI exchange vector plasmids: The p2nd30 vector plasmid, a derivative of pBI121, has an exchange cassette containing a desired gene (*luc*) between oppositely oriented recognition sites (inner *R_s*), *ipt*, recombinase (*R*) genes, and an additional recognition site (outer *R_s*) (Figs. 1 and 2) (5).

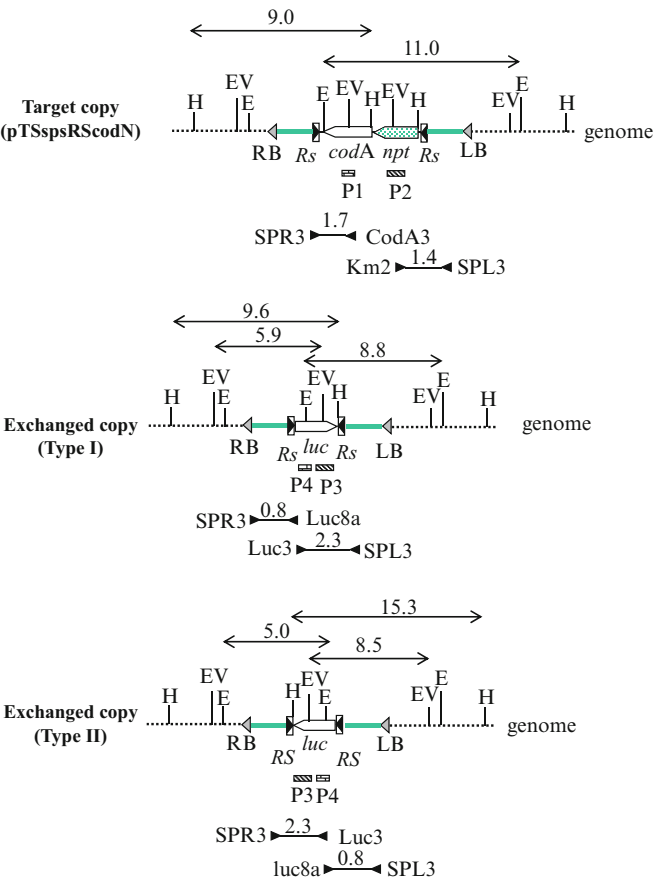


Fig. 2. Diagram of the integrated T-DNA of target and exchanged transgenic plants. The diagram shows the position of probes (P1, P2, P3, P4) for Southern analysis, the size (kb) of fragments detected by the probes, restriction enzyme sites (*E*EcoRI, *E*V EcoRV, *H*HindIII) and the DNA regions amplified with PCR primers (SPR3-CodA3, Km2-SPL3, SPR3-Luc8a, Luc3-SPL3, SPR3-Luc3, Luc8a-SPL3). *codA* cytosine deaminase gene, *npt* neomycin phosphotransferase gene, *gfp* green-fluorescent protein gene, *ipt* isopentenyl transferase gene, *R* recombinase gene, *luc* firefly luciferase gene, *R_s* recombination site, *RB* and *LB* right and left border sequences of a T-DNA.

3. Bacterial strains: *Agrobacterium tumefaciens* LBA4404.
4. YEB medium: 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L of beef extract, 1 g/L of yeast extract, 5 g/L of peptone, 1.2% Bacto-agar, and pH 7.2.
5. Plant materials: *Nicotiana tabacum* cv. Petite Havana SRI plants.
6. MS agar medium: Murashige and Skoog (MS) salts (Icn) and vitamins (Sigma-Aldrich, St. Louis, MO), 20 g/L of sucrose, 0.8% agar (INA agar-BA30; Funakoshi), and pH 5.6.
7. Acetosyringone (Sigma-Aldrich).
8. 6-Benzylaminopurine (BA) solution (Sigma-Aldrich).
9. α -Naphthaleneacetic acid (NAA) solution (Sigma-Aldrich).
10. Carbenicillin (Wako Chemical).
11. Kanamycin (Wako Chemical).
12. Cefotaxime (Wako Chemical).
13. 5-Fluorocytosine (Wako Chemical).
14. FastDNATMKit (Qbiogene).
15. Nylon membranes (Hybond-N, Amersham Pharmacia Biotech).
16. DIG-dUTP, DIG Easy Hyb (hybridization solution), and DIG Wash and Block Buffer Set (Roche Diagnostics GmbH).
17. Cooled CCD LAS-1000 system (Fuji Photo Film).
18. Pica Gene luciferase assay kit (Toyo-Inc., Tokyo, Japan).
19. Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA).
20. Luminescenser-JNR (ATTO, Tokyo, Japan).

3. Methods

We designed the SDI vector system for producing marker-free targeted transgenic plants. The SDI vector system consists of a target vector to introduce a target cassette and an exchange vector to reintroduce an exchange cassette for gene replacement. First, we introduce a target vector into tobacco plants and select transgenic plants with a single copy of a target cassette as target lines. Next, we reintroduce an exchange vector into the target lines and select transgenic plants, in which an exchange cassette is replaced with a target cassette at a predefined chromosomal locus, by using a negative marker.

In this system (Fig. 1), we use the site-specific recombination system (R/Rs), which is derived from the plasmid pSRI of *Zygosaccharomyces rouxii*, and consists of the recombinase (R) and its

recognition sites (*R_s*) (8, 9) (see Note 1), for recombinase-mediated cassette exchange (RMCE). The target cassette contains target genes (*npt* and *codA*) between oppositely oriented recognition sites (*R_s*). The *codA* gene codes for cytosine deaminase, which converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) and is used as a negative marker to select exchanged transgenic plants. Since 5-FU is toxic to plant cells, a transgenic plant with a *codA* gene is inhibited on medium containing 5-FC (10). The exchange vector T-DNA possesses an exchange cassette containing a desired gene (*luc*) between oppositely oriented recognition sites (inner *R_s*). Adjacent to the exchange cassette are *ipt* and recombinase (*R*) genes and an additional recognition site (outer *R_s*). When the exchange cassette aligns with the target cassette on a plant chromosome, recombinase catalyzes double crossover between target *R_s* and exchange inner *R_s* to replace the introduced exchange cassette with the integrated target cassette. As the exchanged transgenic plants have no target gene (*codA*), they can survive on medium containing 5-FC. The *ipt* gene is derived from the Ti-plasmid of *A. tumefaciens* and codes for isopentenyl transferase, which catalyzes cytokinin synthesis. Transgenic plants that contain randomly integrated copies of the exchange vector T-DNA show no rooting ability due to the overproduction of cytokinin from *ipt* gene expression (11, 12). The recombinase can also act on the directly oriented outer *R_s* to remove such randomly integrated copies. The *gfp* gene is located outside the directly oriented outer *R_s* to detect a footprint of their random integration into the genome (see Note 2). The SDI vector system results in single-copy exchange into the target site only in regenerated tobacco at a frequency of 6.3% per treated explant, or 10–17.4% per regenerated line (5).

3.1. Production of Target Lines

3.1.1. Preparation of *A. tumefaciens*

1. Introduce the pTSspsRScodN target vector plasmid into the disarmed *A. tumefaciens* strain LBA4404 by electroporation (13).
2. Select and maintain transformants on YEB agar medium containing 50 mg/L of kanamycin.
3. Inoculate a fresh colony into 10 ml of YEB liquid medium without any antibiotics and culture overnight at 27°C.
4. Dilute the suspension culture to $A_{630}=0.25$ with sterilized water.

3.1.2. Preparation of Plant Materials

1. Select leaves from tobacco plants grown in a growth chamber and surface-sterilize with 1% (v/v) sodium hypochlorite solution for 5 min, followed by three rinses with sterile distilled water.
2. Cut the leaves into approx 8-mm square pieces and immerse in sterilized water until infection is performed.

3.1.3. Infection and Cocultivation

1. Immerse leaf segments in diluted *A. tumefaciens* suspension culture for approx 1 min.
2. Blot dry on sterilized filter paper to remove excess of suspension culture.
3. Place on MS agar medium containing 40 mg/L of acetosyringone for 3 days of cocultivation at 25°C.

3.1.4. Regeneration and Selection of Transgenic Plants

1. Transfer the inoculated leaf segments to MS agar medium containing 1 mg/L benzyladenine, 0.1 mg/L NAA, 500 mg/L of carbenicillin, and 200 mg/L kanamycin, and culture in a culture room at 25–28°C under continuous light 40 mmol/m²S.
2. Transfer to fresh medium every 2 weeks.
3. After 1 month of cultivation, separate the calluses from the leaf segments and transfer to the same medium.
4. After 1 month of cultivation, separate the regenerated shoots from the calluses and transfer to hormone-free MS agar medium containing 200 mg/L Cefotaxime and 200 mg/L kanamycin.
5. Subject normally rooted plants to PCR analysis, propagate the transgenic plants, and maintain in a flask on hormone-free MS agar medium containing 200 mg/L Cefotaxime (see Subheading 3.1.5) (Fig. 2) (see Note 3).
6. After Southern analysis, allow the transgenic plants with a single copy of transgenes to develop completely in a flask (see Subheading 3.1.6) (Fig. 2) (see Notes 4 and 5).
7. Gently pull them out from the agar medium, wash off attached agar from the roots thoroughly with tap water and transfer to soil in pots. Cover the plant and pot with a plastic bag and place in a growth chamber at 25–28°C under 16-h/8-h light (100 mmol/m²S)/dark.
8. After 1 week, gradually open the plastic bag over the next week and then remove.
9. Backcross with wild-type plants (SR1).

3.1.5. PCR Analysis

1. Extract genomic DNA from the leaves of in vitro clones using a FastDNA™Kit, and use for PCR analysis.
2. Perform PCR under standard conditions with 1 min of denaturation, 1 min of annealing, and 2 min of extension at 94°C, 60°C, and 72°C, respectively, for 30 cycles.
3. Resolve the reaction products by electrophoresis in a 1.8% (w/v) agarose gel.

3.1.6. Southern Analysis

1. Isolate genomic DNA from in vitro propagated clones by a modified cetyltrimethyl-ammonium bromide (CTAB) method (14).

2. Digest 10–20 µg of DNA samples with appropriate restriction enzymes, separate on 0.8% (w/v) agarose gel and blot to nylon membranes.
3. Label the probe DNA fragment by PCR using DIG-dUTP following the supplier's instructions.
4. Perform hybridization, washing, and detection using DIG Easy Hyb (hybridization solution) and DIG Wash and Block Buffer Set following the supplier's instructions.
5. Detect the hybridization signals with a cooled charge-coupled device (CCD) system.

3.2. Targeted Transformation

3.2.1. Preparation of *A. tumefaciens*

1. Introduce the p2nd30 exchange vector plasmid into the disarmed *A. tumefaciens* strain LBA4404 by electroporation (13).
2. Select and maintain transformants on YEB agar medium containing 50 mg/L of kanamycin.
3. Inoculate a fresh colony into 10 ml of YEB liquid medium without any antibiotics and culture overnight at 27°C.
4. Dilute the suspension culture is diluted to $A_{630}=0.25$ with sterilized water.

3.2.2. Preparation of Plant Materials

1. Cut the leaves of in vitro transgenic tobacco clones (target lines) into approx 8-mm square pieces and immerse in sterilized water until infection is performed (see Note 6).

3.2.3. Infection and Cocultivation

1. Immerse leaf segments in diluted *A. tumefaciens* suspension culture for approx 1 min.
2. Blot dry on sterilized filter paper to remove excess of suspension culture.
3. Place on MS agar medium containing 40 mg/L of acetosyringone for 3 days of cocultivation at 25°C.

3.2.4. Regeneration and Selection of Exchanged Plants

1. Transfer the inoculated leaf segments to MS agar medium containing 1 mg/L benzyladenine, 0.1 mg/L NAA acid, and 500 mg/L of carbenicillin, and culture in a culture room at 25–28°C under continuous light for 2 weeks.
2. Transfer to MS agar medium containing 1 mg/L BA, 0.1 mg/L NAA, 500 mg/L of carbenicillin, and 200 mg/L 5-fluorocytosine (see Notes 7 and 8).
3. Transfer to fresh medium every 2 weeks.
4. After 1 month of cultivation, separate the calluses from the leaf segments and transfer to the same medium.
5. After 1 month of cultivation, separate the regenerated shoots from the calluses and transfer to hormone-free MS agar

medium containing 200 mg/L Cefotaxime and 200 mg/L 5-fluorocytosine.

6. Subject normally rooted plants to PCR analysis, then propagate and maintain the exchanged transgenic plants in a flask on hormone-free MS agar medium containing 200 mg/L Cefotaxime (see Subheading 3.1.5) (Fig. 2) (see Note 9).
7. After Southern analysis, allow the transgenic plants with only an exchanged copy to develop completely in a flask (see Subheading 3.1.6) (Fig. 2) (see Notes 10 and 11).
8. Gently pull them out from the agar medium, wash off attached agar from the roots thoroughly with tap water and transfer to soil in pots. Cover the plant and pot with a plastic bag and place in a growth chamber at 25–28°C under 16-h/8-h light/dark.
9. After 1 week, gradually open the plastic bag over the next week and then remove.
10. Backcross with wild-type plants (SR1).

3.2.5. Luciferase Assay

1. Surface-sterilize seeds of exchanged transgenic plants with 1% (v/v) sodium hypochlorite solution for 5 min, followed by three rinses with sterile distilled water and place on MS agar medium.
2. One month after cultivation, use three expanded leaves from in vitro T1 transgenic plants for a luciferase assay.
3. Carry out the luciferase assay using the Pica Gene luciferase assay kit (Toyo-Inc., Tokyo, Japan) following the supplier's instructions.
4. Determine the protein concentration of the extract using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA) following the supplier's instructions.
5. Measure the light intensity from 1 µg of the protein extract using a Luminescenser-JNR (ATTO, Tokyo, Japan) for 10 s, and represent it as the relative luciferase activity (RLA/µg) (see Note 12).

4. Notes

1. In the bacteriophage P1 Cre/*lox* system, 34-bp recognition sites in which two 13-bp inverted repeat sequences flank an 8-bp spacer sequence are widely used for site-specific integration and RMCE. We use 400-bp recognition sites derived from the plasmid pSR1 of *Z. rouxii* for the SDI vector system because of their high pairing ability. The plasmid pSR1 contains a pair of the 959-bp inverted repeats, within a 58-bp region on which recombinases catalyze site-specific recombination. This region

contains a 7-bp spacer sequence flanked by two 12-bp inverted repeat sequences. One of these two repeats is flanked by four additional direct repeats with a 4-bp spacer sequence. By PCR, we amplified 400-bp recognition sites containing all these repeats from the 959-bp inverted repeats. As the pairing ability of recognition sites depends on their size and structure, we expect that 400-bp sequences would promote the alignment of a desired gene with a target gene and increase the exchange frequency.

2. We reported that four of eight exchanged transgenic plants contained no *gfp* gene. This indicated that not through the random integration, a desired gene was directly exchanged with a target gene at predefined chromosomal loci by RMCE (5).
3. Two pairs of PCR primers are designed to verify the target cassette (Fig. 2) (5). When the target cassette was integrated into plant genome, both the predicted 1.7-kb and 1.4-kb fragments were amplified with the primer pairs SPR3-codA3 and Km2-SPL3 (Fig. 2). The sequences of the PCR primers were as follows:
 - (a) SPR3: 5'-TTACTTTGCGTTTGTGTACT-3'
 - (b) SPL3: 5'-TGCTTTTTCGTCTGCCATTG-3'
 - (c) codA3: 5'-GTGAAACAACGCGCATGGCA-3'
 - (d) Km2: 5'-AGAGGCTATTTCGGCTATGAC-3'
4. Probes and restriction enzymes are designed to verify the structure of the target cassette by Southern analysis (Fig. 2) (5). In target plants, the predicted 9.0-kb fragment was hybridized with the P1 probe (*codA*) by *Hind*III digestion, and the predicted 11.0-kb fragment was hybridized with the P2 probe (*nptII*) by *Eco*RI digestion (Fig. 2). PCR primers for labeling were as follows:
 - (a) P1 probe: 5'-GTGAAACAACGCGCATGGCA-3' and 5'-CACTTGCAGCATATTTCGCCGT-3'
 - (b) P2 probe: 5'-CCATGATATTTCGGCAAGCAG-3' and 5'-AGAGGCTATTTCGGCTATGAC-3'
5. In the case of our standard tobacco transformation method, we used 64 leaf segments (16 explants per Petri dish) for one routine work, subcultured 60 antibiotic-resistant calluses, and separated less than two shoots from each of independent callus lines. Overall, about 6 months after infection, we obtained 30 normally rooted plants for DNA analysis. About 20% of them contained only a single copy of intact transgenes.
6. Target transgenic lines with high sensitivity to 5-FC were used for retransformation by exchange vectors. Leaves of in vitro clones (target lines) were cut into approx 8-mm square pieces and placed on MS agar medium containing 1 mg/L BA, 0.1 mg/L NAA acid, and 200 mg/L 5-fluorocytosine.

One month after cultivation, we confirmed that leaf segments were inhibited without any callus formation (5).

7. *Agrobacterium*-mediated transformation methods integrate efficiently desired genes into random loci of a plant genome. Therefore, compared with transgenic plants with randomly integrated copies, the proportion of transgenic plants with an exchanged copy is extremely low. To overcome this problem, a strategy in which a selection marker can be activated by introducing a desired gene into a target site is widely used for selection of exchanged transgenic plants (6, 15). At the SDI vector system, transgenic plants with only an exchanged copy are identified as normally rooted plants on the MS agar medium containing 5-fluorocytosine (5-FC) because they lose the *codA* and *ipt* genes (5). We selected 60 calluses resistant to 5-FC and then induced shoots. Almost all shoots (about 80–90%) separated from them showed normal rooting ability. This result indicates that 5-FC negative selection is effective for screening of exchanged transgenic plants.
8. We investigated culture conditions to induce targeting events and selected exchanged transgenic plants by using a negative marker (*codA*). First, just after *Agrobacterium* infection, we placed the infected leaf segments on the medium containing plant hormones and 5-FC for negative selection but could not obtain any exchanged transgenic plants. Therefore, we subcultured them on the medium containing only plant hormones to induce callus formation for 2 weeks before 5-FC negative selection (5). We expect that the promotion effect by the *ipt* gene would play an important role in the efficiency of targeting events. The desired and recombinase genes are introduced together with the *ipt* gene into target lines. The *ipt* gene would increase the frequency of gene targeting events during transformation since cytokinin induces callus formation and proliferates selectively transgenic cells where the desired and recombinase genes are introduced.
9. Two pairs of PCR primers are designed to verify the replacement of an exchange cassette with a target cassette (Fig. 2) (5). When the target cassette was replaced by the exchange cassette, both the predicted 0.8-kb and 2.3-kb fragments were amplified with the primer pairs SPR3-Luc8a and Luc3-SPL3 (Type I), or the predicted 2.3-kb and 0.8-kb fragments were amplified with the primer pairs SPR3-Luc3 and Luc8a-SPL3 (Type II) (Fig. 2). The sequences of the PCR primers were as follows:
 - (a) SPR3: 5'-TTACTTTGCGTTTGTGTACT-3'
 - (b) SPL3: 5'-TGCTTTTTCGTCTGCCATTG-3'
 - (c) Luc3: 5'-CGTTCGGTTGGCAGAAGCTATGAAA-3'
 - (d) Luc8a: 5'-TTTCATAGCTTCTGCCAACCGAACG-3'

10. Probes and restriction enzymes are designed to verify the structure of the exchanged copy at the target locus by Southern analysis (Fig. 2) (5). In Type I exchanged plants, the predicted 8.8-kb or 9.6-kb fragment was hybridized with the P3 probe (*luc*) by *Hind*III or *Eco*RI digestion, respectively, and the predicted 5.9-kb fragment with the P4 probe (*luc*) by *Eco*RV digestion. In Type II exchanged plants, the predicted 5.0-kb or 15.3-kb fragment was hybridized with the P3 probe (*luc*) by *Eco*RI or *Hind*III digestion, respectively, and the predicted 8.5-kb fragment with the P4 probe (*luc*) by *Eco*RV digestion (Fig. 2). PCR primers for labeling were as follows:
 - (a) P3 probe: 5'-CATCACGGTTTTGGAATGTTTACTA-3' and 5'-CGGAGGATTACAATAGCTAAGAATT-3'
 - (b) P4 probe: 5'-CGTTCGGTTGGCAGAAGCTATGAAA-3' and 5'-AGGTGCGCCCCCAGAAGCAATTCG-3'
11. The average task of screening procedures was almost the same as our standard tobacco transformation method. We used 64 leaf segments from each of two target lines (cod23A, B) with a target copy in a heterozygous or homozygous condition, subcultured 60 5-FC-resistant calluses, and separated less than two shoots from each of independent callus lines. Overall, about 6 months after infection, we obtained 40 and 23 normally rooted plants for DNA analysis. About 10% and 17.4% of them contained only a single copy of exchanged transgenes at the target site (5).
12. Conventional transformation methods require a large amount of DNA and RNA analysis to identify transgenic plants with a single copy of desired genes with high expression levels. The SDI vector system enables us to easily identify such valuable transgenic plants simply by using a PCR analysis, and to consistently reproduce the same gene expression level. We reported the introduction of a desired gene (*luc*) into three different target lines (IGN4, 14, 18), which contain a target gene (*gus*) with high expression levels. We compared the luciferase activity of exchanged transgenic plants with that of control transgenic plants containing a single copy of randomly integrated genes (Fig. 3) (3). The exchanged transgenic plants derived from the same target line showed the same level of luciferase activity, which was clearly less variable and higher than that of control transgenic plants. The exchanged plants derived from IGN4 target line showed two times higher levels of luciferase activity than that from IGN14 and 18 target lines. If the same target lines are used as common standard lines, it is possible to precisely compare gene expression levels and optimize gene structures for research and commercial use. Thus, the SDI vector system has great potential for genetic engineering in crops.

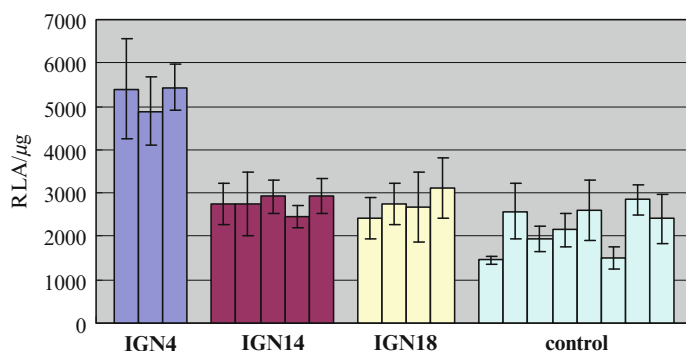


Fig. 3. Luciferase assay of exchanged transgenic plants derived from different target lines. Exchanged transgenic plants from each target plant (IGN4, 14, 18) and control transgenic plants with a randomly integrated copy are backcrossed with wild tobacco plants SR1. The progeny of transgenic plants with an exchanged copy or a randomly integrated copy in heterozygous condition are subjected to luciferase assay. Five T1 plants from each exchanged and control transgenic plant are analyzed, and the average luciferase activity is calculated. Specific activity (RLA/ μ g) is shown on the y-axis as the average value of 5T1 plants.

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Targeting DNA to a Previously Integrated Transgenic Locus Using Zinc Finger Nucleases

Tonya L. Strange and Joseph F. Petolino

Abstract

Targeting exogenously supplied DNA to a predetermined location within a plant genome provides a powerful tool for basic studies of plant gene function and opens up some intriguing possibilities for crop improvement. The induction of double-strand DNA breaks at specific genomic loci via the use of designed zinc finger nucleases (ZFNs) allows for targeted transgene integration. Preintegrating a reporter construct containing a nonfunctional herbicide resistance gene flanked by ZFN binding sites results in a locus capable of being targeted. Retransformation with a corresponding ZFN-expressing cassette and a donor DNA with sequences homologous to the integrated construct and capable of functionalizing the herbicide resistance gene following site-specific integration results in targeted DNA addition. Targeted DNA integration can be confirmed in herbicide-resistant plant cells using PCR analysis.

Key words: Double-strand DNA breaks, Homology-directed repair, Site-directed transgene integration, Zinc finger nucleases

1. Introduction

The ability to target DNA to a previously integrated transgenic locus has value not only for basic expression studies as it relates to transgene “position effect” but also for commercial trait stacking. The ability to create sequence-specific double-strand DNA breaks using zinc finger nucleases (ZFNs) combined with the delivery of donor template DNA homologous to sequences flanking the break site, allows for targeted genome modification, including site-specific transgene integration (1). The following protocol describes a method of gene targeting whereby a target construct containing a nonfunctional herbicide resistance gene—a 3' partial phosphinothricin acetyltransferase (*pat*) fragment—flanked by ZFN binding sites is first stably transformed into the genome of a tobacco cell culture. A second transformation involving

a corresponding ZFN-expression cassette along with a donor template DNA comprising sequences homologous to the integrated construct and a 5' partial *pat* gene sequence (including a promoter region) complementary to the 3' *pat* gene fragment results in the formation of a fully functional herbicide resistance gene and subsequent Bialaphos®-resistant colony formation. In this way, transgenic events resulting from targeted DNA integration following ZFN-mediated, site-specific cleavage can be selected for using herbicide selection. Also provided is a molecular strategy to confirm homology-directed repair at the ZFN target site using PCR analysis involving primers anchored on each of the partial *pat* gene fragments.

2. Materials

2.1. Culture Media

1. "Suspension maintenance": Linsmaier and Skoog basal salts (#L689 PhytoTechnology Labs, Shawnee Mission, KS), KH_2PO_4 (170 mg/L), sucrose (30 g/L), 2,4-D (0.2 mg/L), and thiamine-HCl (0.6 mg/L) adjusted to a pH of 6.0.
2. "Liquid HYG selection" (for target construct integration): "suspension maintenance" medium with the addition of hygromycin (100 mg/L).
3. "Liquid BIA selection" (for targeted transformation): "suspension maintenance" medium with the addition of Bialaphos® (15 mg/L).
4. "Semisolid HYG plating" (for target construct integration): "suspension maintenance" medium with the addition of carbenicillin (500 mg/L) and hygromycin (200 mg/L) and solidified with 8 g/L TC agar (PhytoTechnology, Shawnee Mission, KS).
5. "Semisolid BIA plating" (for targeted transformation): "suspension maintenance" medium with the addition of carbenicillin (500 mg/L) and Bialaphos® (15 mg/L) and solidified with 8 g/L TC agar (PhytoTechnology, Shawnee Mission, KS).
6. "Agro semisolid": Bacto-peptone 10 g/L, yeast extract 10 g/L, sodium chloride 5 g/L, Bacto agar 15 g/L, and, after autoclaving, streptomycin (250 mg/L) and spectinomycin (100 mg/L).
7. "Agro liquid": Bacto-peptone (10 g/L), yeast extract (10 g/L), NaCl (5 g/L), and, after autoclaving, streptomycin (250 mg/L) and spectinomycin (100 mg/L).

2.2. Biological Materials

1. Tobacco suspension culture: BY2 (see Note 1).
2. *Agrobacterium*: strain LBA4404.

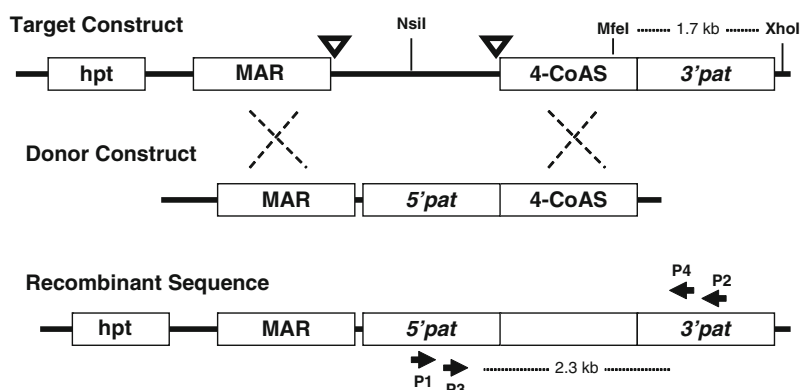


Fig. 1. Schematic of the targeting strategy. Target construct showing the ZFN binding sites (triangles) and the nonfunctional herbicide resistance gene sequence (*3' pat*). Donor construct with homologous sequences (*MAR* and *4-CoAS*) and *5' pat* gene sequence. Recombinant sequence with fully functional *pat* gene and primer sites for nested PCR (arrows).

2.3. Molecular Constructs

1. Target: A hygromycin phosphotransferase (*hpt*) gene (2) can be used as a selectable marker for the initial transformation. The *Nicotiana tabacum* RB7 matrix attachment region (3) and the *Arabidopsis thaliana* 4-coumaroyl synthase intron-1 (Locus At3g21320, GenBank NC 003074) can serve as homologous sequences and a 256-bp partial 3' fragment of the *Streptomyces viridochromogenes pat* gene (4) can be used for subsequent in vitro selection for the second, targeted transformation (Fig. 1). Zinc finger binding sites comprising recognition sequences to which ZFNs can bind are integrated into the target construct (see Note 2).
2. Donor: The same *N. tabacum* RB7 matrix attachment region and *A. thaliana* 4-coumaroyl synthase intron-1 as in the target construct can serve as homologous sequences for homology-directed repair of the ZFN target site upon retransformation of the target events. A full-length *A. thaliana* *ubi10* promoter (4), 299 bp of 5' partial *pat* gene coding sequence (5) that complements the 3' partial *pat* gene in the target sequence and provides in vitro selection following targeted integration.
3. ZFN: A gene encoding a ZFN capable of cleaving a unique 30-bp recognition sequence, following dimerization (see Note 2), in the target construct can be assembled from an archive of in vitro-selected modules (6).

3. Methods

3.1. Maintenance of Tobacco Cultures

1. Subculture cell suspensions every 7 days by adding 0.25 mL PCV to 50 mL of "suspension culture maintenance" medium

and maintain in 150-mL Erlenmeyer flasks on a G10 Gyrotory® shaker (New Brunswick Scientific, Edison, NJ) at 25°C and 125 rpm.

3.2. Generation of Target Reporter Line

1. From a frozen glycerol stock, streak out *Agrobacterium* harboring the target construct onto “Agro semisolid” medium and incubate at 28°C. After 48 h, pick a single colony and inoculate 4 mL of “Agro Liquid” medium (see Note 3) in a 17 mm×100 mm culture tube (Beckman Dickinson, Franklin Lakes, NJ) and place onto a incubator shaker (Model 1540, VWR, West Chester, PA) at 190 rpm at 28°C. After 24 h, inoculate 25 mL of “Agro Liquid” medium (see Note 3) in a baffled Erlenmeyer flask with 5–20 µL of culture, return to the incubator shaker, and grow for 16–20 h to OD₆₀₀ (see Note 4).
2. Divide a flask of a four-day postsubculture suspension into ten to twelve 4 mL aliquots and cocultivate in 100 mm×25 mm Petri dishes with 100 µL *Agrobacterium* harboring the target construct grown overnight to an OD₆₀₀ ~1.2.
3. Wrap dishes with Nescofilm® (Azwel Inc., Osaka, Japan) and incubate at 25°C without shaking for 3 days. Remove excess liquid and replace with 11 mL of “Liquid HYG Selection” medium.
4. Resuspend the *Agrobacterium*-treated cells and dispense 1 mL aliquots onto 100 mm×25 mm plates of “semisolid HYG plating” medium and incubate unwrapped at 28°C in the dark.
5. Individual hygromycin-resistant isolates appear 10–14 days after plating and should be transferred to individual 60 mm×20 mm plates (one isolate per plate) where they are maintained on “semisolid HYG plating” medium as callus on a 14-day subculture schedule until needed for analysis and subsequent retransformation experiments.

3.3. Southern Blot Analysis of Target Reporter Line

1. For each sample, 10 mg of genomic DNA is digested with the appropriate restriction enzymes, separated on a 0.8% agarose gel, and transferred and cross-linked onto a nylon membrane. Specific probes are labeled with P32 dCTP using the Prime-ItRmT Random Primer Labeling Kit (Stratagene, La Jolla, CA) and hybridized to the membrane overnight at 65°C. The hybridized membrane is then washed in graduated increased stringency with the final wash in “SSC solution” (876 mg/L NaCl and 440 mg/L Na citrate dehydrate) with 0.01% SDS at 65°C for 30 min.
2. The membranes are analyzed by a BAS 1500 imaging plate scanner (Fuji Photo Film, Tokyo) or exposed on an X-ray film for a few days at –80°C.

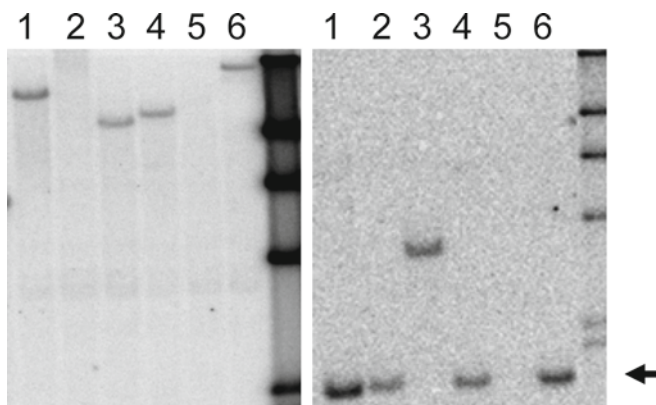


Fig. 2. Southern blot analysis of hygromycin-resistant callus lines. *Left*: *Nsi*I digestion probed with a *pat* gene sequence—lanes 1, 3, 4, and 6 displaying single copy of the target construct. *Right*: *Mfe*I/*Xho*I digestion probed with a *pat* gene sequence—lanes 1, 2, 4, and 6 showing the expected 1.7-kb fragment (arrow).

3. To determine the copy number and intactness of the target sequence (see Note 5), genomic DNA is digested with *Nsi*I (which has a single recognition sequence within the target construct) or *Mfe*I/*Xho*I, respectively (Fig. 1), prior to being probed with a *pat* gene sequence (Fig. 2).

3.4. Retransformation of Target Reporter Line

1. Transgenic cell cultures containing a single, full-length integrated copy of the target sequence can be selected and used to reinitiate suspension cultures by placing 250–500 mg of callus tissue into 40–50 mL of “liquid HYG selection” medium and subculturing every 7 days as described above (see Note 6).
2. *Agrobacterium*-mediated transformation of the target cell cultures is performed as described above using 50 μ L each of two *Agrobacterium* strains: one harboring the donor DNA and a second harboring the ZFN construct.
3. Following cocultivation, the cells are resuspended in “liquid BIA selection” medium and plated out on “semisolid BIA plating” medium.
4. Individual Bialaphos[®]-resistant isolates appear 2–4 weeks after plating (Fig. 3).
5. Resistant isolates can be transferred to individual 60 mm $\times$ 20 mm plates (one isolate per plate) and maintained on “semisolid BIA plating” medium as callus on a 14-day subculture schedule until needed for analysis.

3.5. PCR Analysis of Targeted Integration

1. Genomic DNA is extracted from tobacco callus using DNeasy 96 Plant kit (Qiagen, Valencia, CA) and quantified using PicoGreen ds DNA Quantitation kit (Molecular Probes,

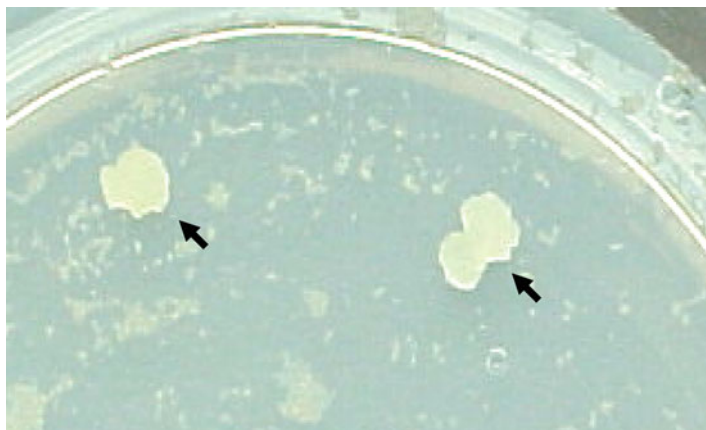


Fig. 3. Bialaphos[®]-resistant isolates (*arrows*) following retransformation of target events with donor DNA and ZFN-expressing constructs.

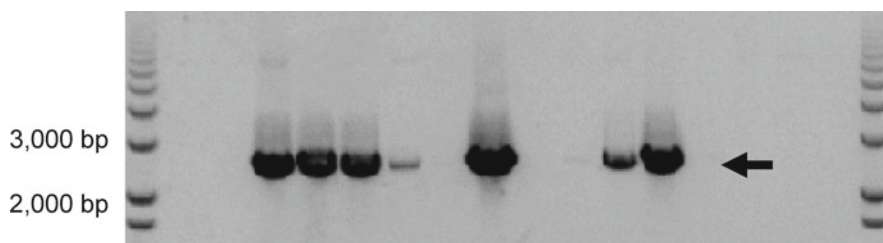


Fig. 4. PCR amplification of a 2.3-kb recombinant product in Bialaphos[®]-resistant cultures.

Eugene, OR). An aliquot of 2 μ L of extracted genomic DNA is checked through agarose gel electrophoresis to ensure the DNA quality.

2. Herbicide-resistant, putative recombinant events can be analyzed by PCR initially with primer pair of P1 (TAAGGATCC AACCATGGCTTCTCC) and P2 (AGATCTGGGTAAGTGG CCTAACTG). To further enhance the sensitivity of this assay, a nested PCR can then be performed using primer pair of P3 (TACCCTTGGTTGGTTGCTGAGGTT) and P4 (GAAGGC CTATAACAGCAA CCACAG).
3. The primers used to confirm targeted DNA integration anchor to the 5' and 3' ends of the *pat* coding sequence and amplify a 2.3-kb fragment spanning the two partial *pat* gene sequences—only if recombination between the donor and target sequences occur (Fig. 4).
4. To further confirm the sequence of the PCR products from recombinant events, the PCR fragment can be purified from the gel using the QIAquick gel extraction kit (Qiagen),

subcloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) then sequenced using the Dye Terminator Cycle Sequencing Kit.

4. Notes

1. Obtained from Jun Ueki, Japan Tobacco, Iwata, Shizuoka, Japan.
2. Zinc finger protein moiety = RSDDLKDNSNRIKRSDALS VDNANRTK binds GCTCAAGAACATGTGGTCATGTTC TTGAGC as a homodimer.
3. The antibiotics used in these media depend on the selectable markers used on the binary plasmids.
4. Set up multiple overnight cultures by inoculating with 5, 10, and 20 μL to ensure that one is at the desired OD_{600} when convenient.
5. Any genomic DNA digestion and Southern blot analysis that determines copy number and intactness of the integrated sequence would suffice. An event containing a single, completely intact copy of the construct is preferred for retransformation.
6. Two weeks prior to *Agrobacterium* transformation, switch to "Suspension Culture Maintenance" medium (without hygromycin) for culture maintenance.

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Double-Strand Break-Induced Targeted Mutagenesis in Plants

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Abstract

Double-strand breaks are very potent inducers of DNA recombination. There is no recombination between DNA molecules unless one or two DNA strands are broken. It has become feasible to introduce double-strand breaks at specific chromosomal loci by using dedicated, redesigned endonucleases with altered DNA-binding specificities. Such breaks are mainly repaired by error-prone nonhomologous recombination pathways in somatic cells, thus frequently producing mutations at the preselected chromosomal sites. Although the art and science of reengineering protein properties have been advancing quickly, an empirical validation of new endonucleases in a particular experimental environment is essential for successful targeted mutagenesis experiments. This chapter presents methods that were developed for a comprehensive evaluation of the DNA-binding and DNA-cutting activities of homing endonucleases in maize cells; however, they can be adopted for similar evaluation studies of other endonucleases and other plant species that are amenable for *Agrobacterium*-mediated transformation.

Key words: Targeted mutagenesis, Double-strand break, Endonuclease, Transgenic plants, Maize, *Zea mays* L.

1. Introduction

Double-strand breaks (DSB) are introduced throughout the genome in meiosis. Their repair leads to the formation of cross-overs essential for a proper segregation of homologous chromosomes. This is a regulated process that involves a number of interacting proteins with Spo11 actually making a cut (1). In somatic cells, DSBs can be produced by abiotic stresses (the most common are irradiation and toxic chemicals). DSBs also emerge as a result of malfunction of the DNA replication process—for example, stalling of replication forks (2). Their repair frequently leads to mutations, since nonhomologous end-joining (NHEJ) seems to

be the prevalent mechanism of DNA repair in somatic cells (3). In this context, the introduction of endonucleases that produce chromosomal DSBs may be considered as a potent mutagenic procedure. If the introduced endonuclease shows DNA-binding specificity, the process may be referred to as targeted mutagenesis.

There are two distinct approaches to the production of site-specific endonucleases: a fusion of DNA-binding domain to an endonuclease DNA-cutting domain and protein engineering of existing, natural site-specific endonucleases—in particular, homing endonucleases (4, 5). The fusion of zinc-finger DNA-binding domains to the *FokI* endonuclease DNA-cutting domain is a preferred combination for making artificial fusion endonucleases (6, 7), while the I-*CreI* homing endonuclease from *Chlamydomonas reinhardtii* has frequently been used as a core protein scaffold for engineering DNA-binding specificities of homing endonucleases (8). Other options include TAL (transcription activator-like) DNA-binding domains fused to *FokI* or alternative homing endonucleases such as I-*SceI*, I-*AniI*, and I-*MsoI* (9–11). Both strategies have delivered promising results by introducing double-strand breaks/mutations/foreign DNA insertions at specific chromosomal loci in a number of higher eukaryotic species (12–16).

Whether reengineered endonucleases are selected by high-throughput screening procedures (17), produced by rational design (11), or a new DNA-binding specificity is identified by a combination of such procedures (18), it is essential that the DNA-binding specificity and the overall double-strand break-making activity is validated *in vivo* conditions, preferably, the cellular environment of a target organism to be mutagenized. It has become evident that the results of *in vitro* assays do not correlate well with the performance of redesigned endonucleases in genetic transformation experiments. Cytotoxicity of the engineered endonucleases (possibly due to the relaxed DNA-binding specificity) and the reduced DNA-breaking activity are two essential parameters that may negatively affect the final outcome of mutagenesis (19, 20). Therefore, it is prudent and highly recommended to validate the performance of a redesigned endonuclease before engaging in a time-consuming and expensive targeted mutagenesis activity.

Of note, the plant mutagenesis experiments may not require highly specific and active redesigned endonucleases as opposed to the potential applications of such reagents in mammalian or human gene therapy applications. Highly efficient plant transformation procedures can compensate for a diminished activity of redesigned endonucleases, and off-target mutations due to the relaxed DNA-binding specificity may be eliminated in the next generations of mutagenized plants. Still, it is important to make sure that the transformed cells survive expression of redesigned endonucleases, and the frequency of double-strand breaks at the intended target site needs to be sufficient for a dependable identification of the

mutant plants. The protocols presented in this chapter are designed to help in selecting the best performing endonucleases for a particular genetic engineering application.

Since the first observations on the mutagenic effects of DSB repair in plants produced either by homing endonucleases (I-*SceI*) or artificial zinc-finger endonucleases (21, 22), their applications have extended into endogenous chromosomal sites and redesigned endonucleases with altered DNA-binding specificities (13–16, 23–25). It seems that it is now feasible and practical to precisely introduce mutations at preselected chromosomal loci. At this stage of technology development, the mutations that depend on the nonhomologous end-joining DNA repair (gene knockouts by altering coding sequences, eliminating receptor proteins, inactivation of regulatory gene expression pathways that rely on DNA-protein interactions) are more easily obtained. However, there are reports on the other types of genome modifications that include a foreign DNA insertion at specific chromosomal locations and homologous recombination between the incoming DNA and chromosomal loci—gene targeting (26). The reliability of such procedures will depend on the quality of redesigned endonucleases that need to maintain their DSB activity and DNA-binding specificity, regardless of the changing preferences for new recognition sequences. The protocols presented in this chapter help to validate the modified endonucleases for subsequent applications. Although not tested for zinc-finger endonucleases and any cells other than BMS suspension cultures, it is anticipated that the thermal shift assay should work for any DNA-binding protein, and it should be possible to adopt the endonuclease activity assay for any plant suspension cultures that are amenable to *Agrobacterium* transformation.

The recent publication of a collaborative work between Keck School of Medicine of University of Southern California and Sangamo Biosciences, Inc. that led to the targeted mutagenesis of the *CCR5* receptors in human cells by zinc-finger nucleases making the cells resistant to HIV-1 infection demonstrates the power and potential of double-strand break-based technologies (15). The underlying mechanisms of double-strand break repair appear to be universal in all higher eukaryotic organisms, and the different types of endonucleases seem to be comparably effective in making double-strand breaks. The consistent results produced by different endonucleases operating in different organisms underscore the general utility of targeted mutagenesis induced by double-strand breaks.

1.1. Thermal Shift Assay

The thermal shift assay (see Note 1) is used here for the DNA-binding specificity evaluation of redesigned endonucleases. Originally, the assay was developed for analysis of protein-ligand interactions, mainly for the drug discovery programs (27). We extended its application on protein-DNA interactions. Since the

procedures can be adjusted for a high-throughput screening, the thermal shift assay can also be used as a powerful tool for the analysis of interactions between amino acids of engineered endonucleases and a large number of potential new recognition sequences.

The formation of an endonuclease-DNA complex (any substrate or ligand in that matter) results in a change of protein stability in solution. Stronger, more specific binding to the target sequence results in a more stable protein-DNA complex. The shift in the midpoint temperature of transition (unfolding), ΔT_m , of such complexes is used as a measure of DNA-binding affinity. By comparing the relative ΔT_m measurements of a protein in complex with different DNA target sequences, it is possible to evaluate DNA-binding specificity. The midpoint temperature of transition is estimated by inspecting denaturation curves of the endonuclease-DNA complex within a range of test temperatures. The protein-unfolding process is monitored using the SYPRO Orange fluorogenic dye, which fluorescence is quenched in the presence of native (folded) proteins. After reaching the midpoint temperature, fluorescence starts to decrease probably due to the precipitation of protein-dye complexes (Fig. 1).

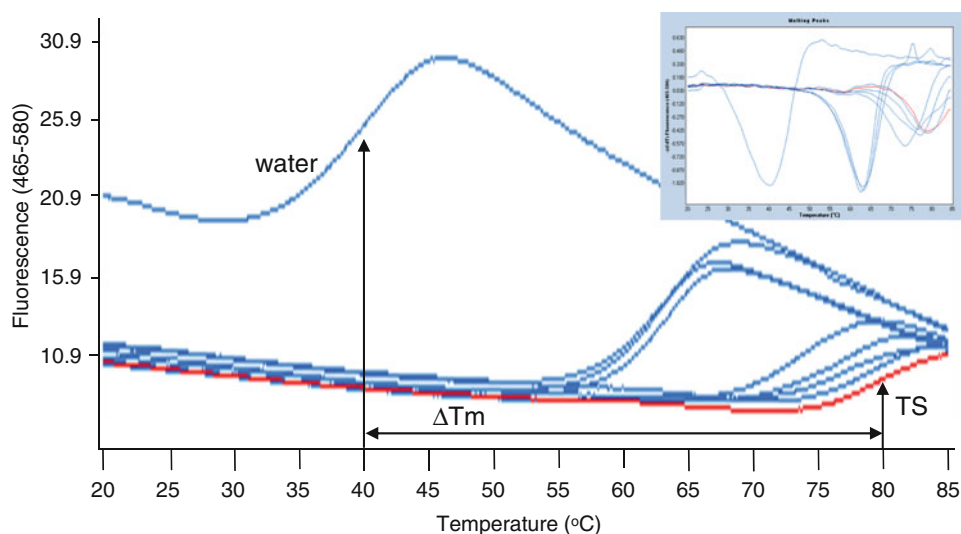


Fig. 1. Changes in fluorescence of an endonuclease protein solution containing the SYPRO® Orange dye over different temperature ranges. The inflection point of a denaturation curve (*arrowed lines pointing up*) is used to determine the melting temperature of the unbound protein (water) and the protein bound to its cognate target site (TS, *red line*). The difference in the melting temperatures (ΔT_m) is used as an indicator of the DNA-binding specificity. The additional melting curves (*blue lines*) show a fluorescence of endonuclease protein solutions in the presence of alternative recognition sequences. The *inset* shows a derivative presentation of the melting curves that is used to calculate melting temperatures (the midpoint temperature of the protein-unfolding transition).

1.2. Endonuclease Activity Assay (see Notes 2 and 3)

While the thermal shift assay provides information on the DNA-protein interactions, it does not validate the overall DSB-making activity of redesigned endonucleases. It is assumed that an endonuclease needs to bind to the target DNA sequence for making double-strand breaks, but the DNA binding alone does not imply that the bound sequence will be cut. *In vitro* incubation of endonucleases with DNA target sites can indicate whether the modified protein retains DSB activity, but such data are not always predictive of *in vivo* results.

The proposed endonuclease activity assay is designed to monitor double-strand breaks produced inside a plant cell by relying on the endogenous nonhomologous end-joining DNA repairs that result in mutations at the target sites. The target DNA sequences are delivered and replicated inside the plant cells together with an endonuclease to be validated. Subsequently, the target sequences are recovered in *E. coli* and then subjected to sequence analysis.

It seems that in maize somatic cells, like in other higher eukaryotic organisms, the double-strand break repair process is mutagenic (Fig. 3). Short deletions and insertions are the most frequently observable outcome of such repairs (12–15, 21, 22). Therefore, the endonuclease activity assay that is based on counting the number of mutagenized target sites should deliver more accurate results as compared to the methods based on the less-frequent homologous recombination reactions.

2. Materials

2.1. Chemicals and Equipment for Thermal Shift Assay

1. SYPRO® Orange Protein Gel Stain 5,000× concentrate (Sigma).
2. PB buffer—25 mM Tris-Cl pH 8.0, 100 mM NaCl, 5 mM MgCl₂, and 5 mM EDTA.

DNA binding may, or may not, be affected by the enzymatic activity of a tested endonuclease. Incubations without metal ions in the buffer solution should provide conditions for testing such options.

3. Complementary single-stranded target DNA molecules (Sigma) diluted in PB buffer and annealed to achieve 50 μM 60-mer DNA oligo solutions. The 22-bp recognition sequences are flanked by 16-bp and 22-bp random sequences to the left and right side, respectively.
4. Endonuclease protein is produced in BL21 (DE3) cells transformed with the pET-21a bacterial expression vector (Novagen, EMD Chemicals, Gibbstown, NJ) and purified on nickel-charged metal-chelating Sepharose column.

5. The thermal shift assay is conducted in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN).
6. White LightCycler® 480 Multiwell 384 plates with sealing foils are used for the incubations (Roche Diagnostics, Indianapolis, IN).

2.2. Other Chemicals, Media, and Equipment

1. Corn cell suspension media (CCSM).
4.3 g/L Murashige and Skoog basal salt mixture (PhytoTechnology Laboratories), 5.0 mL/L MS vitamins stock solution (Sigma), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 30 g/L sucrose.
2. N6 media with acetosyringone (N6AC).
4.0 g/L Chu (N₆) Basal Salts Mix (Sigma), 1.0 mL/L 1,000× Eriksson's Vitamin Mix, 0.5 mg/L thiamine-HCl, 1.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.690 g/L L-proline, 68.5 g/L sucrose, 36.0 g/L glucose, and 0.1 mM acetosyringone, pH 5.2.
3. Liquid media with spectinomycin for *Agrobacterium* (LMS).
10.5 g/L potassium phosphate dibasic, 4.5 g/L potassium phosphate monobasic anhydrous, 1 g/L ammonium sulfate, 0.5 g/L sodium citrate dihydrate, 2 g/L sucrose, 1 mM magnesium sulfate, and 50 mg/L spectinomycin dihydrochloride.
4. Master plate with spectinomycin for *Agrobacterium* (MPS).
3 g/L potassium phosphate dibasic, 1 g/L sodium phosphate monobasic anhydrous, 1 g/L ammonium chloride, 0.3 g/L magnesium sulfate heptahydrate, 0.15 g/L potassium chloride, 11.4 mg/L calcium chloride, 2.5 mg/L ferrous sulfate heptahydrate, 5 g/L glucose, 15 g/L agar, and 50 mg/L spectinomycin dihydrochloride.
5. Solid N6 media with acetosyringone (N6ACP).
4.0 g/L Chu (N₆) Basal Salts Mix (Sigma), 1.0 mL/L 1,000× Eriksson's Vitamin Mix, 0.5 mg/L thiamine-HCl, 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.690 g/L L-proline, 30.0 g/L sucrose, 0.85 mg/L silver nitrate, 0.1 nM acetosyringone, and 8.0 g/L agar (Sigma), pH 5.8.
6. Solid media with 2,4-D and carbenicillin.
4.0 g/L Chu (N₆) Basal Salts Mix (Sigma), 1.0 mL/L 1,000× Eriksson's Vitamin Mix, 0.5 mg/L thiamine-HCl, 1.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.690 g/L L-proline, 30.0 g/L sucrose, 0.85 mg/L silver nitrate, 0.5 g/L

- 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, 100 mg/L carbenicillin, and 8.0 g/L agar (Sigma), pH 5.8.
7. Solid LB media with kanamycin.
25 g/L LB Broth (Miller) Powder Growth Media, 15 g/L Bacto agar, and 50 mg/L kanamycin sulfate, pH 7.0.
 8. 2XYT Broth with kanamycin.
31 g/L 2XYT Broth (Miller) Powder and 50 mg/L kanamycin sulfate, pH 7.0.
 9. 2XYT Broth with spectinomycin.
31 g/L 2XYT Broth (Miller) Powder and 100 mg/L spectinomycin dihydrochloride, pH 7.0.
 10. BMS (black Mexican sweet) maize cell suspension.
 11. *Agrobacterium* cointegrate strain.
 12. Sterile 250-mL glass flasks with caps.
 13. 50-mL polypropylene conical tubes (BD Falcon).
 14. Sterile 50-mL serological pipettes.
 15. Sterile 35-mL serological pipettes.
 16. 100- μ m nylon cell strainer (BD Falcon).
 17. 50-mm Filter unit (Nalgene).
 18. Disposable cuvettes for use in spectrophotometer.
 19. Multiwell 6-well tissue culture plate (BD Falcon).
 20. Parafilm.
 21. Glass fiber filter discs, grade 691, 5.5 cm (VWR).
 22. Wide-bore pipette tip, 1,000 μ L (Axygen Scientific).
 23. DNeasy Plant Mini Kit (QIAGEN).
 24. Mortar and pestle.
 25. Electroporation 1-mm-gap cuvettes (BTX).
 26. MegaX DH10BTM T1^R ElectrocompTM cells (Invitrogen).
 27. 96-well Styrene plates (Thermo Scientific).
 28. Platform shaker.
 29. Incubator.
 30. Spectrophotometer.
 31. Gene Pulser II, capacitance extender, and pulse controller (Bio-Rad).
 32. Centrifuge (3,600 $\times g$).
 33. Microcentrifuge (17,900 $\times g$).
 34. Laminar flow hood.

3. Methods

Once per week, subculture cell suspension by mixing 15 mL established cell suspension with 30 mL CCSM in a sterile 250-mL flask.

3.1. Thermal Shift Assay (see Note 1)

1. Dilute the SYPRO Orange stock solution 500-fold with PB buffer to obtain 10× SYPRO Orange solution.
2. Dilute endonuclease protein preparation to 4 μM in the 10× SYPRO Orange solution. Depending on the sensitivity of the instrument used for fluorescence measurements, optimize the protein and dye concentrations from 0.5 to 8 μM for protein and 5× to 20× for SYPRO® Orange.
3. For each reaction, add 8 μL of 4 μM protein (32 pmoles) in 10× SYPRO Orange solution and 2 μL of 50 μM target DNA in PB buffer (100 pmoles). Use 2 μL of water instead of the DNA target solution in a mock treatment. The final concentration of protein is 3.2 μM , and the final DNA target concentration is 10 μM .
4. Heat the plate from 20°C to 99°C with a heating rate of 0.06°C per second and measure the fluorescence at Ex/Em: 490/580 nm. Determine the inflection point (change of the melting curve from concave to convex) by the software provided with the instrument.

3.2. BMS Maintenance and Preparation

1. Media and labware should be sterile. Work in the laminar flowhood. Use sterile handling techniques to avoid contamination.
2. Refresh the cultures 2–3 days after subculturing by adding an additional 30 mL CCSM.
3. Keep cell suspensions at 28°C on a platform shaker set to 150 rpm.
4. On day of BMS transformation, let BMS cells from one 75-mL suspension settle to the bottom of flask and remove as much supernatant as possible with pipette.
5. Transfer up to 30 mL of remaining suspension into a 50-mL tube and, again, let cells settle.
6. Remove another 25 mL of the liquid media, if possible.
7. To remove remaining liquid media, pipette slowly from very bottom of the tube, not allowing cells in, until air bubbles appear in the pipette.
8. Add 40 mL fresh, room temperature N6AC media to the cells.

9. Mix gently and transfer cell suspension to a sterile 250-mL flask.
10. Shake suspension on a platform shaker set to 150 rpm for at least 1 h before BMS cell transformation.
11. After shaking, let BMS cells settle to bottom of flask and remove 20 mL supernatant from the top.
12. Pipette remaining cell suspension into a 100- μ m cell strainer inside a filter unit.
13. Using a vacuum, filter the liquid off, continually adding more BMS cell suspension until strainer is filled with cells.
14. There should now be 5 packed cell volume (pcv); transfer cells to new 50-mL tube.
15. Add N6AC media to bring total volume to 10 mL. The final concentration of the BMS cell suspension should be 0.5 pack cell volumes of BMS/mL.

3.3. Preparation of *Agrobacterium* Cell Suspension

1. One day before transformation, start *Agrobacterium* liquid culture in a 50-mL tube by mixing 30 mL LMS media with a loopful of bacteria from a master plate or use 100 μ L of an *Agrobacterium* glycerol stock.
2. Incubate bacterial suspension for at least 18 h before transformation at 28°C on a platform shaker set to 150 rpm.
3. After overnight incubation, centrifuge the liquid culture for 10 min at 3,600 $\times g$ at room temperature.
4. Pipette off supernatant and resuspend bacterial pellet in 5 mL N6AC media.
5. Make a 10 $\times$ dilution of the liquid suspension in a 1-mL cuvette and measure the optical density at 550 nm with a spectrophotometer, using 1 mL of N6AC media as a blank.
6. Plug the optical density (OD) into the following formula to calculate the final volume that would yield an approximate concentration of 5.0×10^8 cells/mL: $4.9 \times \left[e^{(1.755 \times \text{LN}(\text{OD}) + 21.77)} \times 10 \right] / 5.0 \times 10^8$.
7. Add N6AC media to the working *Agrobacterium* suspension to reach final volume.

3.4. Transformation of Maize BMS Cells

1. Combine 2 mL *Agrobacterium* suspension (5.0×10^8 cells/mL) and 2 mL BMS cell suspension (0.5 pcv/mL) in one well of 6-well tissue culture plate.
2. Close plate with lid and seal with parafilm.
3. Incubate the cocultivation mixture for 3 h at 28°C on a platform shaker set to 150 rpm.
4. After incubation, set plate at an angle and let BMS cells settle to bottom of plate and remove as much supernatant as possible.

5. Place 10–15 50- μ L drops of the remaining cell suspension onto a sterile glass fiber filter disc.
6. Transfer filter disc to N6ACP media plate.
7. Incubate plate(s) at 28°C for 24 h.
8. Transfer filter discs to media with 2, 4-D and carbenicillin plate.
9. Six days after transformation, harvest cells and extract total DNA.

3.5. DNA Isolation from BMS Cells

1. Harvest all BMS cells from one filter disc and transfer to mortar.
2. Add 600 μ L buffer AP1 and 6 μ L RNaseA (100 mg/mL) stock, from Qiagen's DNeasy Purification Kit, to BMS cells.
3. Homogenize cells with pestle for ~2 min until mixture is runny.
4. Follow Qiagen's DNeasy Purification of Total DNA from Plant Tissue Mini Kit from the 10 min incubation at 65°C step through elution of DNA with 100 μ L AE Buffer.

3.6. *E. coli* Transformation and Plasmid Sequencing

1. Place electroporation cuvette and DH10 β TM-T1^R ElectrocompTM cells on ice for 5 min.
2. When thawed, add 25 μ L DH10 β TM cells and 100 ng total DNA from transformed BMS cells to the electroporation cuvette.
3. Replace cap and gently tap cuvette on bench to mix and remove any air bubbles.
4. Place cuvette in electroporator holder and electroporate with conditions set to 2.0 kV, 200 $\mu\Omega$, and 25 μ F.
5. Spread 25 μ L of electroporated *E. coli* on prewarmed to 37°C LB media with kanamycin plate.
6. Incubate plate at 37°C overnight.
7. Fill a 96-well, v-bottom clear plate with 100 μ L 2XYT broth with kanamycin per well.
8. Make an identical plate with 100 μ L 2XYT broth with spectinomycin per well.
9. Pick 96 colonies and inoculate both plates with each individual colony.
10. Seal plates with PCR plate sealer and incubate at 37°C overnight on a shaker set to 250 rpm.
11. Use 2 μ L of each overnight culture that grew in kanamycin media, but not spectinomycin media, as a sample for analysis by PCR and sequencing or qPCR.
12. Perform PCR and sequencing according to preferred protocol.

13. We have been using REDExtract-N-Amp PCR Mix (Sigma) and 0.25 mM primers that flank target site region on the plasmid DNA to produce 400 bp PCR products. PCR products were purified using either Unifilter GF/F filter plates (Whatman) or AcroPrep Omega 30 K filter plates (PALL).
14. DNA sequencing was performed by BigDye Terminator chemistry on ABI 3700 capillary-sequencing machines (Applied Biosystems). Each sample contained approximately 100 ng of the PCR products and 6.4 pmol sequencing primer.

3.7. Endonuclease Activity Assay (see Notes 2 and 3)

1. Use the BMS (black Mexican sweet) maize suspension culture for the infection with *Agrobacterium*. *Agrobacterium* T-DNA delivers an endonuclease expression cassette, the yeast FLP site-specific recombinase, the WDV (wheat dwarf virus) replication-associated protein (replicase), and the endonuclease target recognition sequences (Fig. 2). The two latter components are flanked by the FLP recombination targets (*FRT*). The endonuclease target sites are excised by FLP in maize cells, and the circular DNA fragment is replicated by the WDV replicase (the origin of replication is embedded into the WDV promoter (*LIR*)). If the endonuclease makes a double-strand break at the target site, its repair may produce mutations.

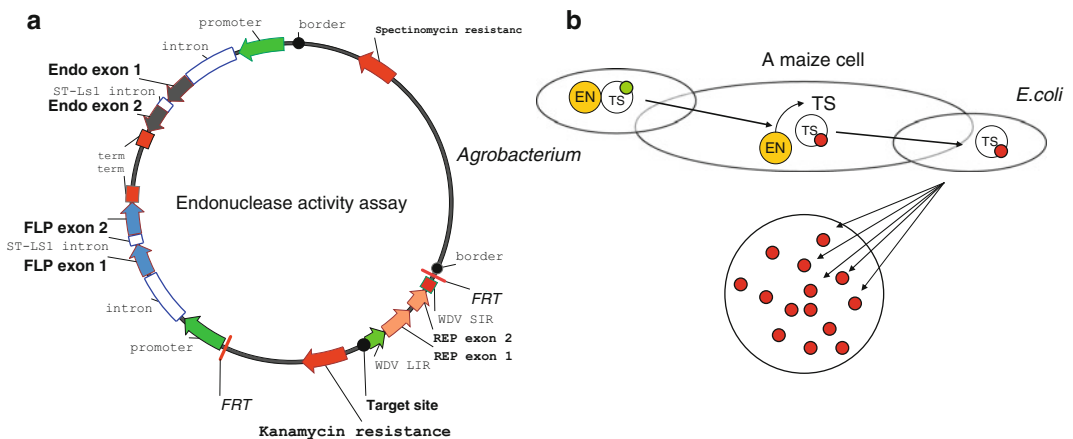


Fig. 2. Endonuclease activity assay. (a) The key genetic elements delivered to maize cells by *Agrobacterium* (the DNA fragment delimited by borders). The expression units for an endonuclease (Endo) and the FLP site-specific recombinase (FLP) contain introns in the coding sequences in order to prevent their expression in *Agrobacterium*. Target site and the WDV replicase expression cassette (REP) are flanked by the *FRT* sites. A circular excision product of the FLP-mediated recombination is replicated by the viral replication system. This replicating fragment also contains the kanamycin resistance gene (kanamycin) and the bacterial origin of replication (Cole1 ori) for the proliferation of this fragment in *E. coli*. (b) A diagram illustrating the concept of transient endonuclease activity assay. An endonuclease (EN) and target sites (TS) are both delivered to maize cells. Double-strand breaks at the target sites are repaired and recovered in *E. coli*. The recovered target sites are sequenced to identify mutations introduced by the nonhomologous end-joining DNA repair pathway.

GAGCTCTGGTACCAAATATACCTCACACGTACGCGTA	
GAGCTCTGGTACCAAATATACC	ACACGTACGCGTA
GAGCTCTGGTACCAAATAT	ACACGTACGCGTA
GAGCTCTGGTACCAAATATA	ACACGTACGCGTA
GAGCTCTGGTACCAAATATACCT	CACGTACGCGTA
GAGCTCTGGTACCAAATATAC	CACGTACGCGTA
GAGCTCTGGTACCAAATA	CACGTACGCGTA
GAGCTCTGGTACCA	CACGTACGCGTA
GAGCTCTGGTACCAAATATA	ACGTACGCGTA
GAGCTCTGGTACCAA	CGTACGCGTA
GAGCTCTGGTACCA	CGTACGCGTA
GAGCTCTGGTACCAAATATACCTCAC	ACGCGTA
GAGCTCTGGTACCAAATATACC	C CGTACGCGTA

Fig. 3. Endonuclease activity generates mutations at the *lig34* target site (the recognition target site is highlighted in blue, the core region in red). Empty spaces indicate deletions.

2. Extract total DNA from the infected BMS cells 6 days after transformation and use for electroporation of *E. coli*. Use the bacterial colonies grown on media with kanamycin, but not on media supplemented with spectinomycin, for PCR or plasmid DNA preparations and sequence the target sites on these plasmids.
3. Use the ratio of target sites that contain mutations to the total number of sequenced target sites as a measure of an endonuclease double-strand break activity. A sample of mutations at the *lig34* target site is shown in Fig. 3.

4. Notes

1. The thermal shift assay provides a relatively straightforward validation of the sequence specificity of redesigned endonucleases. It allows a clear distinction between general, unspecific DNA-binding and specific target recognition (Fig. 4; compare the *T_m* values for the random DNA sequence and the *lig34* target sequence). The experimental error of *T_m* determination is estimated to be ±0.1°C.

The assay is sensitive enough to distinguish changes in DNA-binding specificity caused by 1 or 2 substitutions in the target sequence (Fig. 4; compare *T_m* values for the recognition sequences 3, 4, and 5). A contribution to the DNA-binding specificity of nucleotides that are not directly contacted by amino acids can also be detected by this assay (Fig. 4; the recognition sequences 2 and 3 differing in the core region sequence GTGA). The core region of the I-*CreI* target site

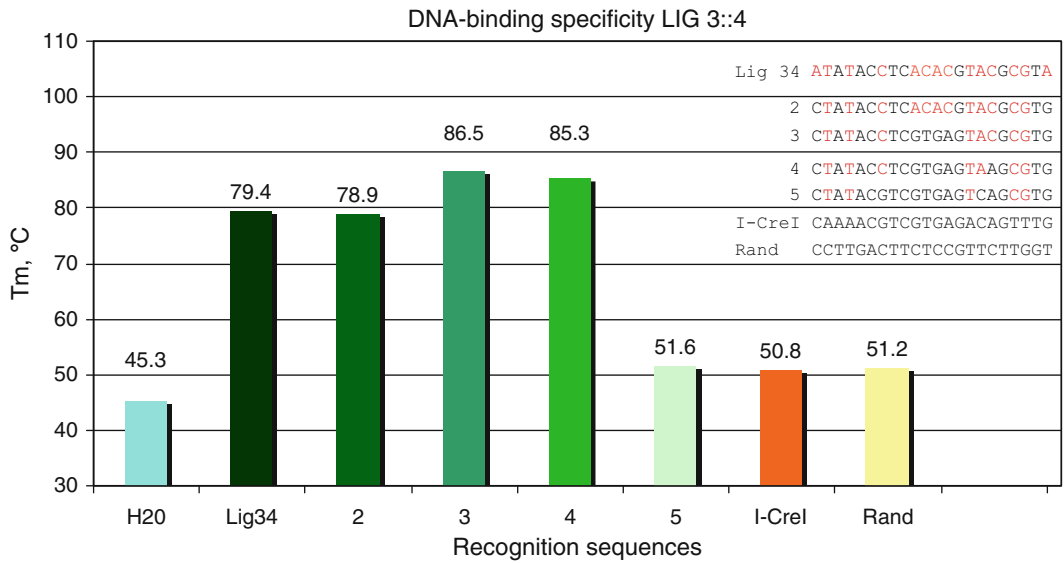


Fig. 4. The midpoint temperatures of transition of the LIG 3::4 endonuclease incubated in the presence of different DNA target sequences. The *lig34* and *I-CreI* recognition sequences represent the intended target site for the LIG 3::4 endonuclease and the original wild-type target site of *I-CreI*, respectively. The different nucleotides at particular positions of the target sequence are *highlighted in red (inset)*. The melting temperature of the LIG 3::4 protein without any DNA molecules in the solution is shown as H₂O, while the *Rand treatment* represents incubation with a 60-nucleotide-long random sequence oligonucleotide.

seems not to be involved in making specific protein-DNA contacts; however, it may affect secondary DNA structures that are important in the DNA-protein interactions (28).

2. The key challenge of any endonuclease redesign project is to change the recognition sequence and still preserve protein DNA-binding specificity to that target sequence. If it is not accomplished, the common feature of redesigned proteins is that the relaxed DNA-binding specificity leads to promiscuity in the target site selection and an increased cytotoxicity of reengineered endonucleases. However, the decrease in DNA-binding requirements can also generate more double-strand breaks at the designated target site, thus compensating for a potential loss of catalytic activity caused by amino acid substitutions. It is important to take such factors into consideration while selecting an endonuclease for stable transformation experiments.

Two potential target sites were selected for the introduction of double-strand breaks at a 13-kbp DNA fragment of maize genome (TS A and TS B). The wild-type coding sequence of the *I-CreI* homing endonuclease was modified in order to produce four variants (Endo A1, Endo A2, Endo B1, and Endo B2) with altered DNA-binding properties (two variants

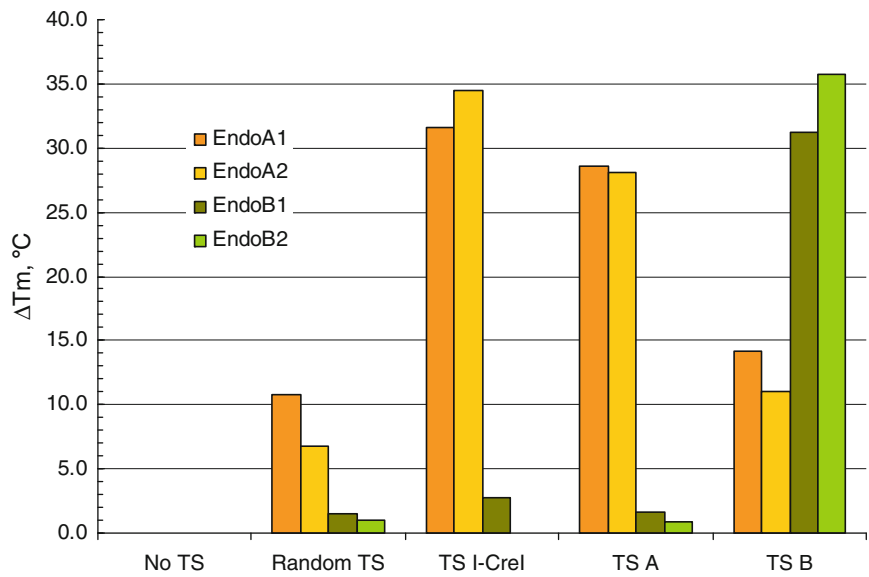


Fig. 5. DNA-binding specificity as estimated by the ΔT_m parameter for four redesigned endonucleases: Endo A1, Endo A2, Endo B1, and Endo B2. TS A and TS B are designated recognition sequences for the two classes of endonucleases, Endo A1,2 and Endo B1,2, respectively. The panel TS I-*CreI* represents ΔT_m of the endonuclease complexes with the original wild-type I-*CreI* target site (TS I-*CreI*). The panel random TS illustrates unspecific DNA binding to the random sequence 60-mer oligonucleotides.

per target site). The DNA-binding specificities of these endonucleases are summarized in Fig. 5.

It seems that the Endo A's DNA-binding specificities were relaxed as a result of amino acids substitutions designed to change its recognition sequence. The most remarkable feature of the Endo A's endonucleases was their retention of DNA-binding specificity toward the original wild-type I-*CreI* recognition sequence (panel TS I-*CreI*). The binding of random DNA sequences was also enhanced by amino acid substitutions (Random TS) as well as binding to the other target site (TS B). In contrast, the Endo B's endonucleases showed an acceptable pattern of DNA-binding specificities that discriminate between the original I-*CreI* target site and its new recognition sequence (TS B). The Endo B's endonucleases should be the preferred choice of the enzyme for stable transformation experiments.

- 3. A sample of results generated by the endonuclease activity assay is presented in Table 1. We found that the native I-*CreI* homing endonuclease did not produce mutations at the LIG 3::4 designated target site—*LIG-I*, and the enhanced version of LIG 3::4, LIG 3::4++, did not produce mutations at the wild-type I-*CreI* target site. This is consistent with the thermal

Table 1
Targeted mutagenesis in transient endonuclease activity assay and stable transformation experiments. The mutation rates are expressed as the percentage of clones or T₀ plants that acquired mutations

Endonuclease activity assay					Stable transformation		
Target site	Endonuclease	# Clones sequenced	# Mutations	Mutation rate	T ₀ plants	Mutant T ₀ plants	Mutation rate
<i>-CreI</i>	LIG 3::4 ++	380	0	0			
<i>LIG-I</i>	I-CreI	380	0	0			
<i>LIG-I</i>	None				62	0	0
<i>LIG-I</i>	LIG 3::4	637	3	0.5	781	10	1.3 ^a
<i>LIG-I</i>	LIG 3::4 +	353	1	0.3	249	7	2.8
<i>LIG-I</i>	LIG 3::4 ++	237	56	23.6	75 ^b	27	36.0
<i>TS A</i>	Endo A1	64	1	1.6			
<i>TS A</i>	Endo A2	165	5	3.0			
<i>I-CreI</i>	Endo A2	47	14	29.8			

^aData adapted from ref. 14

^bOnly T₀ plants with a single-copy LIG 3::4++ integration are included in the calculations

shift assay results indicating that the LIG 3::4 endonuclease does not bind the I-*CreI* target site in a specific manner.

In contrast, the Endo A2 endonuclease produced mutations in about 30% of the I-*CreI* target sites. This observation is again consistent with the thermal shift assay result showing DNA-specific binding of Endo A2 to the wild-type I-*CreI* target site. Interestingly, the Endo A2 protein binds with similar affinity to the designated *TS A* target site in the thermal shift assay, but its DSB activity is substantially diminished (about 3% mutation rate in the endonuclease activity assay) as compared to the wild-type I-*CreI* site. This observation indicates that the DNA-binding affinity alone cannot determine the overall performance of a particular endonuclease. We find a good correlation between the mutation rates in the endonuclease activity assay and the mutagenesis rates of the stable transformation experiments that deliver mutant plants. This is illustrated by the performance of three versions of the LIG 3::4 endonuclease. Although the LIG 3::4++ version evidently produces more double-strand breaks than the LIG 3::4 version in both experimental setups, it is important to note that the targeted mutagenesis rates in the range of 1% seem to be satisfactory for the production of mutant plants. This statement may not be true for the other potential applications of the double-strand break technology such as site-specific integrations and gene targeting.

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Part VII

Metabolic Engineering and Pharming

Chapter 33

Combinatorial Genetic Transformation of Cereals and the Creation of Metabolic Libraries for the Carotenoid Pathway

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Abstract

Combinatorial nuclear transformation is used to generate populations of transgenic plants containing random selections from a collection of input transgenes. This is a useful approach because it provides the means to test different combinations of genes without the need for separate transformation experiments, allowing the comprehensive analysis of metabolic pathways and other genetic systems requiring the coordinated expression of multiple genes. The principle of combinatorial nuclear transformation is demonstrated in this chapter through protocols developed in our laboratory that allow combinations of genes encoding enzymes in the carotenoid biosynthesis pathway to be introduced into rice and a white-endosperm variety of corn. These allow the accumulation of carotenoids to be screened initially by the colour of the endosperm, which ranges from white through various shades of yellow and orange depending on the types and quantities of carotenoids present. The protocols cover the preparation of DNA-coated metal particles, the transformation of corn and rice plants by particle bombardment, the regeneration of transgenic plants, the extraction of carotenoids from plant tissues, and their analysis by high-performance liquid chromatography.

Key words: Combinatorial transformation, Metabolic pathway, Metabolic engineering, Carotenoid pathway, Particle bombardment, Transgenic plants, Metabolite profiling

1. Introduction

Combinatorial nuclear transformation is a novel method for the rapid production of populations of transgenic plants wherein each member of the population carries a random sample from a collection of input transgenes (1). We developed this system to dissect and modify the carotenoid metabolic pathway in corn, although in principle, it can be applied to any metabolic pathway or any other complex phenotype requiring the coordinated expression of

multiple genes. To demonstrate the principle, we transferred five genes encoding enzymes in the carotenoid biosynthesis pathway, in addition to the selectable marker gene *bar* (encoding phosphinothricin acetyltransferase, which confers resistance to the herbicide glufosinate), into the elite white corn inbred M37W, which is deficient for endosperm carotenoid synthesis. Each gene was controlled by a different, endosperm-specific promoter. We recovered a diverse population of transgenic plants expressing different enzyme combinations and showing distinct metabolic phenotypes that allowed us to identify and complement rate-limiting steps in the pathway and to demonstrate competition between β -carotene hydroxylase and β -carotene ketolase for substrates in four sequential pathway steps (1).

The combinatorial system is potentially very useful because it allows complex pathways to be dissected in a single transformation experiment. Studying and engineering secondary metabolism in plants is difficult when (as is often the case) the pathways are complex, with multiple branches, enzymes that participate in multiple steps and compete for substrates, complex feedback-control mechanisms, and subcellular compartmentalization of enzymes and substrates (2, 3). Traditionally, it has been necessary to dissect such pathways by laboriously modulating the levels of each enzyme, either by introducing a transgene to achieve overexpression or attenuating expression using antisense RNA, cosuppression/RNA interference, or the expression of an intracellular antibody directed against the enzyme. Such methods are often frustrated by the complex way in which pathways are regulated, so it is becoming clear that multistep engineering will be required to dissect complex pathways, i.e., strategies in which partial or complete pathways are overexpressed using multiple transgenes, or where different enzymes are overexpressed or attenuated in the same plant lines (3–5).

Unfortunately, any scheme for multigene engineering that is both comprehensive and rationally designed would quickly become impractical if each transgene combination were deliberately introduced. For example, consider a simple and linear pathway with just four enzymatic steps. A comprehensive dissection in which each enzyme was in turn overexpressed and attenuated, and where all possible different combinations of upregulated and downregulated enzymes were assembled, would require 225 different plant lines (Fig. 1). In each case, at least 10–20 individual transformants would be required to ensure that the sought after combination was recovered because transgene integration is a random process, and even if a transgene is integrated, its expression might be affected by epigenetic phenomena. The number of plants required for higher transgene numbers would quickly become unsupportable because of the exponential increase in the number of different variants required (5, 6).

We used as a model system the South African elite white corn inbred M37W, which lacks carotenoids in the endosperm due to the absence of the enzyme phytoene synthase (PSY1) (Fig. 2) (7, 8).

This provided a blank canvas upon which to study the metabolic capabilities of the input transgenes in different combinations, providing a rapid readout of carotenoid accumulation based on endosperm colour. However, there is nothing in principle preventing the use of other corn varieties and other plants to achieve the same goals, as long as the endogenous levels of metabolites are used as the baseline and fluctuations caused by different transgene combinations are judged against this reference. Therefore, we have recently extended the combinatorial transformation concept to rice, and protocols in this chapter are provided for both corn and rice plants.

2. Materials

2.1. Plant Material

1. The transformation protocol for corn (*Zea mays* L.) plants (Subheading 3.1) is optimized for cultivar M37W, but other cultivars can also be used (9). Plants should be maintained in a climate-controlled growth chamber until fully acclimated, with a day/night temperature of 28/24°C, 70%/85% humidity, and a 14-h photoperiod. The plants should then be transferred to a greenhouse (24–28°C, 70% humidity) until seeds are available for collection.
2. The transformation protocol for rice (*Oryza sativa* L.) plants (Subheading 3.2) is suitable for many different cultivars (10). Plants should be grown under the same conditions described above for corn.

2.2. Handling Plants

1. Controlled environment room.
2. Plant culture room with illumination system.
3. Laminar flow hood.
4. Stereomicroscope with light (for corn and rice transformation).
5. Fine forceps (two pairs) and scalpel.
6. 30°C/37°C incubator.
7. Whatman 3MM filter paper.
8. Rotary shaker.
9. Petri dishes.
10. Falcon tubes.
11. Mortar and pestle.
12. Parafilm.
13. Sterile, double-distilled water.
14. 70% (v/v) Ethanol.

15. 20% and 50% (w/v) Sodium hypochlorite solutions.
16. Autoclave and/or 0.2 μm sterile filter.
17. pH meter.

2.3. Growth Media and Additives

2.3.1. Basic Components

1. MS (Murashige and Skoog) salts (11) with minimal organics (see Note 1).
2. Sucrose (tissue culture grade).
3. Gellan gum (e.g., Gelrite or Phytagel) (see Note 2).
4. Agar (see Note 2).
5. Mannitol (Sigma).
6. Sorbitol (Sigma).
7. KOH for pH adjustment.

2.3.2. Additives and Stock Solutions (see Note 3)

1. Hygromycin B (Sigma). Stock solution: 50 mg/mL in H_2O (see Note 4).
2. Phosphinothricin (PPT) supplied as glufosinate-ammonium powder (Sigma). Stock solution: 10 mg/mL in H_2O (see Note 4).
3. 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma). Stock solution: 5 mg/mL in ethanol.
4. 6-Benzylaminopurine (6-BAP) (Sigma). Stock solution: 1 mg/mL in DMSO.
5. α -Naphthaleneacetic acid (NAA) (Sigma). Stock solution: 1 mg/mL in H_2O .

2.3.3. Media Composition

All media should be prepared immediately before use with ultra-pure water ($>18 \text{ M}\Omega/\text{cm}$) and autoclaved or filter-sterilized (0.2 μm). Heat-sensitive components (selective agents, phytohormones) must be added after cooling to below 50°C.

Corn Transformation

1. Basic MS medium for corn (see Note 5). Make up MS salts and minimal organics as shown in Table 1 or prepare from a ready-mixed powder (see Note 1).
2. MSHc medium (MS with phytohormones for corn): basic MS medium for corn supplemented with 20 g/L sucrose, 4 g/L gellan gum, and 2.5 mg/L 2,4-D.
3. MSOc (MS osmoticum medium for corn): MSHc supplemented with 0.2 M mannitol and 0.2 M sorbitol.
4. MSSc (MS selection medium for corn): MSHc supplemented with 3 mg/L PPT.
5. MSRc (MS regeneration medium for corn): basic MS medium for corn supplemented with 30 g/L sucrose, 4 g/L gellan gum, 10 mg/L 6-BAP, 0.25 mg/L 2,4-D, and 3 mg/mL PPT.

Table 1
MS salts and minimal organics (11). All stocks should be stored at 4°C. The macroelements, microelements, and iron can be added before autoclaving, but the organic compounds should be added after cooling

Ingredients	MS
<i>Macroelements</i> (mg/L)	
Make up as a 10× stock in sterile distilled water	
CaCl ₂ ·2 H ₂ O	440
KH ₂ PO ₄	170
KNO ₃	1,900
MgSO ₄ ·7H ₂ O	370
<i>Microelements</i> (mg/L)	
Make up as a 100× stock in sterile distilled water	
CoCl ₂ ·6H ₂ O	0.025
CuSO ₄ ·5H ₂ O	0.025
H ₃ BO ₃	6.2
KI	0.83
MnSO ₄ ·4H ₂ O	22.3
Na ₂ MoO ₄ ·2H ₂ O	0.25
ZnSO ₄ ·7H ₂ O	8.6
<i>Iron</i> (mg/L)	
Make up as 100× stock in sterile distilled water	
FeSO ₄ ·7H ₂ O	27.8
Na ₂ EDTA·2H ₂ O	37.3
<i>Organic components</i> (mg/L)	
Make up as 100× stock in sterile distilled water. Filter-sterilize	
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Glycine	2
Myoinositol	100
Nicotinic acid	0.5
Casein hydroxylate	1,000

6. MSRMc (MS rooting medium for corn): basic MS medium for corn supplemented with 30 g/L sucrose, 4 g/L gellan gum, and 3 mg/L PPT. This medium is similar to MSRc but contains no phytohormones.

Rice Transformation

1. Basic MS medium for rice (see Note 6). Make up MS salts and minimal organics as shown in Table 1 or prepare from a ready-mixed powder (see Note 1).
2. MSHr (MS with phytohormones for rice): basic MS medium for rice supplemented with 30 g/L sucrose, 5 g/L gellan gum, and 2.5 mg/L 2,4-D.

3. MSOr (MS osmoticum medium for rice): basic MS medium for rice supplemented with 30 g/L sucrose, 3 g/L gellan gum, 2.5 mg/L 2,4-D, and 0.2 M mannitol.
4. MSSr (MS selection medium for rice): MSHr supplemented with 30 mg/L hygromycin B.
5. MSRr (MS regeneration medium for rice): basic MS medium for rice supplemented with 0.2 M maltose, 4 g/L gellan gum, 3 mg/L 6-BAP, 0.5 mg/L NAA, and 30 mg/L hygromycin B.
6. MSRMr (MS rooting medium for rice): basic MS medium for rice supplemented with 10 g/L sucrose, 3 g/L gellan gum, 2 g/L agar, and 30 mg/L hygromycin B. This medium contains no phytohormones.

2.4. Materials for Transformation

1. Stock solution of plasmids carrying the metabolic transgenes (see Notes 7 and 8).
2. Stock solution of plasmid DNA carrying selectable marker gene (see Note 9).
3. Particle accelerator gun and accessories (Bio-Rad or home-made devices) (see Note 10).
4. Gold particles (see Note 11).
5. 100 mM Spermidine.
6. 25% PEG (MW 8,000).
7. 100% Ethanol.
8. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
9. 2.5 M CaCl₂.
10. Sonicator.

2.5. Analysis of Carotenoids

2.5.1. Equipment

1. ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) linked to a PDA 2996 detector (Waters, Milford, MA, USA). MassLynx™ software version 4.1 (Waters, Milford, MA, USA).
2. UNICAM UV/Vis spectrophotometer UV-2.
3. Mortar and pestle.
4. Separation funnel.
5. Gravity funnel.
6. Steel tube.
7. Quartz cuvette.
8. Whatman Grade No. 40 quantitative filter paper (Schleicher & Schuell).
9. Durapore polyvinylidene fluoride (PVDF) membrane filters (Millipore, Bedford, MA, USA).

2.5.2. Chemicals

All chemicals are analytical grade unless otherwise specified. Aqueous solutions must be prepared with ultrapure water. Solvents should be filtered and degassed before use (we recommend 0.22- μ m Durapore PVDF Membrane Filters).

1. β -Carotene (Sigma–Aldrich Fine Chemicals).
2. Zeaxanthin (Sigma–Aldrich Fine Chemicals).
3. Lutein (Sigma–Aldrich Fine Chemicals).
4. Lycopene (Sigma–Aldrich Fine Chemicals).
5. Astaxanthin (Sigma–Aldrich Fine Chemicals).
6. Methanol (B.T. Baker).
7. Acetonitrile (B.T. Baker).
8. Hexane (B.T. Baker).
9. 2-Methoxy-2-methylpropane (methyl *tert*-butyl ether; B.T. Baker).
10. Ethoxyethane (diethyl ether; B.T. Baker).
11. Oxacyclopentane (tetrahydrofuran; B.T. Baker).
12. Propanone (acetone; B.T. Baker).
13. NaCl (Panreac).

3. Methods

3.1. Transformation of Corn Plants

The manipulation of seeds, explants, and callus should be carried out using sterilized tools and equipment in a laminar flow hood to avoid microbial contamination. We recommend that all glassware is cleaned with double-distilled water (no detergents), covered with aluminum foil (see Note 12) and sterilized at 180°C for 20 h.

3.1.1. Preparation of Corn Explants for Bombardment

1. Collect cobs from corn plants in the greenhouse. Immature embryos are required for the following protocol (see Note 13).
2. Wash cobs in 70% ethanol and then 20% sodium hypochlorite for 20 min on a rotary shaker.
3. Transfer work to the laminar flow hood.
4. Rinse cobs at least three times in sterile distilled water.
5. Excise the immature embryos using fine forceps.
6. Place the embryos, scutellum-uppermost on Petri dishes containing MSHc medium.
7. Incubate the plates in darkness at 24°C for 4–6 days.
8. Four hours before bombardment, transfer embryos to MSOc medium (see Note 14).

*3.1.2. Preparation of
DNA-Coated Gold Particles
for Bombardment*

1. Prepare high-quality plasmid DNA at a concentration of 10 mg/mL (see Note 8).
2. Mix 10 mg of gold particles with 5 µg of the selectable marker plasmid and 15 µg of each plasmid carrying the carotenogenic transgene. Add TE buffer to 100 µL. Vortex for 30 s.
3. Add 100 µL of 100 mM spermidine to protect the DNA during the precipitation process. Vortex for 30 s.
4. Add 100 µL of 25% PEG (MW 8,000) and vortex for 30 s.
5. Slowly add 100 µL of 2.5 M CaCl₂ with continuous vortexing. When all the CaCl₂ has been added, vortex for a further 10 min.
6. Centrifuge at 5,000×*g* for 30 s and discard supernatant.
7. Wash with 200 µL 100% ethanol and centrifuge at 5,000×*g* for 30 s.
8. Add 100 µL of 100% ethanol and sonicate briefly to break up clumps of particles.
9. Spot 5–10 µL of the suspension onto the center of a carrier disc and allow to air dry.

*3.1.3. Bombardment Using
Bio-Rad Helium Gun (PDS
1000/He)*

1. Follow manufacturer's guidelines for loading carrier disc into the barrel of the helium gun.
2. Place plant material in the targeting chamber.
3. Bombard plant material at 900–1,300 psi (see Note 15).
4. Bombard embryos twice with a 4-h interval between bombardments (maintain the embryos in darkness at 24°C on MSO during the interval).

*3.1.4. Selection and
Regeneration of Transgenic
Corn Plants*

1. Transfer bombarded embryos to MSHr medium. Incubate in darkness at 24°C for 2 days.
2. For the induction of transgenic embryogenic callus, transfer embryos to MSSc medium and incubate in darkness at 24°C for 2 weeks.
3. Subculture any callus recovered under selection at 2-week intervals on fresh MSSc medium in darkness at 24°C (see Note 18).
4. At the end of the third round of selection, transfer explants to MSRC medium for regeneration. Incubate plates at 24°C under low light (100 µmol/m²/s) for the first 10 days and stronger light (130 µmol/m²/s) for the next 10 days, with an 18-h photoperiod.
5. For rooting, transfer regenerating callus to MSRMc medium under the stronger light conditions described above.

6. When transgenic corn plantlets are approximately 3–4 cm long, transfer them to soil (see Note 18).
7. Maintain transgenic corn plants in a growth chamber with day/night temperature and humidity conditions of 28/24°C and 70/85%, with a 14-h photoperiod for 3 months. Then transfer to greenhouse conditions (24–28°C, 70% humidity, sunlight, and a 14-h photoperiod).

3.2. Transformation of Rice Plants

As discussed for corn, carry out the manipulation of seeds, explants, and callus using sterilized tools and equipment in a laminar flow hood to avoid microbial contamination. We recommend that all glassware is cleaned with double-distilled water (no detergents), covered with aluminum foil (see Note 12) and sterilized at 180°C for 20 h.

3.2.1. Preparation of Rice Explants for Bombardment

1. Collect seeds from rice plants in the greenhouse. Immature embryos can be used for transformation, but we recommend the following protocol that uses mature seed-derived callus as it is more efficient (see Note 16).
2. Dehusk the seeds using a mortar and pestle and collect them in a 50-mL Falcon tube (see Note 17).
3. Sterilize the seeds in 100% ethanol for 2 min, rinse three times in distilled water (optional), and then wash in 50% sodium hypochlorite for 20 min.
4. Transfer to a laminar flow hood and rinse the seeds three times with sterile distilled water and blot dry on Whatman 3MM paper.
5. Place the seeds on MSHr medium (20 seeds per Petri dish) and check for contamination daily. Discard contaminated seeds and transfer the remainder to fresh dishes.
6. After 1 week in culture, excise mature embryos from the surviving seeds and place them, scutellum-uppermost, on MSHr medium.
7. Incubate the plates in darkness at 24°C for 1 day.
8. Four hours before bombardment, transfer embryos to MSOr medium (see Note 14).

3.2.2. Bombardment Using Bio-Rad Helium Gun (PDS 1000/He)

1. Prepare DNA-coated gold particles as described in Subheading 3.1.2.
2. Follow manufacturer's guidelines for loading carrier disc into the barrel of the helium gun.
3. Place plant material in the targeting chamber.
4. Bombard plant material at 900–1,300 psi (see Note 15).

5. Bombard embryos twice with a 4-h interval between bombardments (maintain the embryos in darkness at 24°C on MSOr during the interval).

3.2.3. Selection and Regeneration of Transgenic Rice Plants

1. Transfer bombarded embryos to MSHr medium. Incubate in darkness at 24°C for 1 day.
2. For the induction of transgenic embryogenic callus, transfer embryos to MSSr medium and incubate in darkness at 24°C for 2 weeks.
3. Subculture any callus recovered under selection at 2-week intervals on fresh MSSr medium in darkness at 24°C (see Note 18).
4. After the third round of selection, transfer explants to MSRr medium for regeneration. Incubate plates at 24°C under low light (100 $\mu\text{mol}/\text{m}^2/\text{s}$) for the first 10 days and stronger light (130 $\mu\text{mol}/\text{m}^2/\text{s}$) for the next 10 days, with an 18-h photoperiod.
5. For rooting, transfer regenerating callus to MSRMr medium under the stronger light conditions described above.
6. When transgenic rice plantlets are approximately 10-cm long, transfer them to soil (see Note 19).
7. Maintain transgenic rice plants in a growth chamber with day/night temperature and humidity conditions of 28/24°C and 70/85%, with a 14-h photoperiod for 3 months. Then transfer to greenhouse conditions (24–28°C, 70% humidity, sunlight, and a 14-h photoperiod).

3.3. Carotenoid Analysis by HPLC

3.3.1. Carotenoid Extraction

1. Freeze-dry seeds before commencing extraction.
2. Grind the dry samples into fine powder using a mortar and pestle, washing the equipment between samples to avoid cross-contamination.
3. Transfer 50–100 mg of each sample into a steel tube (see Note 20).
4. Add 15 mL (1:1 v/v) tetrahydrofuran:methanol and cover with aluminum foil.
5. Transfer to a water bath and incubate at 60°C for 20 min.
6. Cool at room temperature for 10–15 min.
7. Transfer the extract to a separation funnel. Line a gravity funnel with filter paper then filter the carotenoid extract into the separation funnel to remove starch.
8. Transfer filter back to the steel tube, add 5 mL acetone, and incubate for 5 min.
9. Pour the remaining extract carefully in the separation funnel.

10. Add 15 mL (9:1 v/v) hexane:diethyl ether. Invert the separation funnel to mix and vent to remove vapor.
11. Add 20 mL of saturated NaCl and repeat the mixing and venting step.
12. Allow the layers to separate (~1 min).
13. Remove lower layer.
14. Add saturated NaCl as above and repeat the mixing and venting step.
15. Allow the layers to separate and again remove the lower layer.
16. Dry the organic phase under N₂ in a block at 37°C until the volume is 5 mL.
17. Transfer 1 mL of (9:1 v/v) hexane:diethyl ether to a cuvette and use this to set the baseline absorbance of the spectrophotometer at 450 nm.
18. Measure the absorbance of 1 mL of the organic phase from the sample.
19. Pour the sample back into the tube and leave it under N₂ for further drying.
20. When the sample is completely dry, fill the tube with argon gas and close tightly with a rubber stopper.
21. Cover the tube with aluminum foil and store at -80°C.

3.3.2. Chromatography

1. For liquid chromatography, the solvent is a 70:50:30 (v/v) mixture of acetonitrile, acetone, and methanol. Filter the solvent through a 0.22- μ m Durapore PVDF Membrane Filter before use. Each extract sample for analysis should be dissolved in 300 μ L of the filtered solvent.
2. Perform the separation of lutein and zeaxanthin using a YMC C₃₀ carotenoid 3- μ m, 2.0 $\times$ 100-mm HPLC column (Waters, Milford, MA) with a mobile phase comprising solvent A (methanol:water, 80:20, v/v) and solvent B (100% methyl *tert*-butyl ether).
3. Warm the sample to 25°C and inject 10 μ L into the column, which should also be thermostatically maintained at 25°C. Set the flow rate to 0.3 mL/min.
4. Set the gradient program as follows: initial conditions 97% solvent A and 3% solvent B for 6 min; change with a linear gradient to 62% solvent A and 38% solvent B in 1 min, hold for 8 min; change with a linear gradient to 32% solvent A and 68% solvent B in 1 min, hold for 10 min; then return to initial conditions in 4 min, followed by equilibration for 5 min.
5. Perform the separation of all other carotenoids using a reversed phase column (ACQUITY UPL® BEH 300 Å C₁₈, 1.7 μ m,

- 2.1 × 150 mm; Waters, Milford, MA), with a mobile phase comprising solvent A (acetonitrile:methanol, 70:30, v/v) and solvent B (100% water).
6. Warm the sample to 25°C and inject 5 µL into the column, which should be thermostatically maintained at 32°C. Set the flow rate to 0.4 mL/min.
 7. Set the gradient program as follows: initial conditions 80% solvent A and 20% solvent B for 2 min; change with linear gradient to 100% solvent A in 1 min, hold for 9 min; then return to initial conditions in 0.1 min, followed by equilibration for 2 min.
 8. Monitor absorbance at 450 nm.
 9. Identify carotenoids by monitoring the following parameters: order of elution from the column, ultraviolet and visible spectra, and the spectral fine structure (%III/II) (12) with literature data (13) and with that of the authentic standards. Also, use those standards for quantitation in combination with the extinction coefficients (13).

4. Notes

1. The ingredients for these basic media are listed in Table 1, although, ready-mixed powders can be purchased from many suppliers (e.g., Gibco-BRL, Flow Labs, Sigma).
2. Two gelling agents are used in the rice transformation protocol because various studies have shown that the “stiffness” of the medium affects callus growth and the efficiency of shoot and root formation (14).
3. Some chemicals may require warming to 37°C and/or long stirring before they dissolve. Before use, briefly vortex all stock solutions.
4. The choice of selective agent depends on the selectable marker used for transformation. Hygromycin B is preferred in rice because kanamycin is not as effective. Hygromycin B is used with the marker *hpt* (from *Klebsiella* spp., encoding hygromycin B phosphotransferase). PPT selection is used with the markers *bar* or *pat*, both of which encode phosphinothricin acetyltransferase.
5. Immature M37W corn embryos must be cultured on semisolid nutritive media based on MS salts (9). The basic MS medium for corn contains the salts and organic compounds listed in Table 1. Several variants are required for different stages of growth, transformation, and regeneration, all of which contain sucrose and a gelling agent (gellan gum) and some of which

contain additional components. The sucrose and gelling agent should be mixed with the basic medium prior to autoclaving, but labile reagents such as the phytohormones and selection agents should be added when the autoclaved medium has cooled below 50°C. It is useful to make up basic MS salts and minimal organics with 4 g/L gellan gum and 20 g/L sucrose for the MSHc, MSOc, and MSSc media and to make a second batch with 4 g/L gellan gum and 30 g/L sucrose for the MSRC and MSMRC media. Add the sucrose first, and before adding the gelling agent, adjust the pH to 5.8 with 1 M KOH. MSHc is the basic MS salts and organics recipe supplemented with 20 g/L sucrose, 4 g/L gellan gum, and 2.5 mg/L 2,4-D to promote callus growth. An osmoticum medium (MSOc) is used prior to bombardment, and this is MSHc supplemented with 0.2 M mannitol and 0.2 M sorbitol (see Note 14). A selection medium (MSSc) is required to isolate transgenic callus expressing the *bar* marker gene, and this is MSHc supplemented with 3 mg/L PPT. The regeneration medium (MSRC) is the basic MS salts and organics recipe supplemented with 30 g/L sucrose, 4 g/L gellan gum, 0.25 mg/L 2,4-D, 10 mg/L 6-BAP, and 3 mg/L PPT to encourage the growth of transgenic shoots. The rooting medium (MSRMc) is similar to MSRC but contains no phytohormones.

6. Mature rice embryos are cultured on media based on MS salts (15, 16). The basic MS medium for rice contains the salts and organic compounds listed in Table 1. Several variants are required for different stages of growth, transformation, and regeneration, all of which contain a carbon source (sucrose or maltose) and a gelling agent (gellan gum or gellan gum with agar; see Note 2) and some of which contain additional components. The sugar and gelling agent should be mixed with the basic medium prior to autoclaving, but labile reagents such as the phytohormones and selection agents should be added when the autoclaved medium has cooled below 50°C. Add the sugar first, and before adding the gelling agent, adjust the pH to 5.8 with 1 M KOH. MSHr is the basic MS salts and organics recipe supplemented with 30 g/L sucrose, 5 g/L gellan gum, and 2.5 mg/L 2,4-D to promote callus growth. An osmoticum medium (MSOr) is used prior to bombardment, and this is similar to MSHr, but contains a slightly lower concentration of gelling agent (3 g/L gellan gum; see Note 2) plus 0.2 M mannitol (see Note 14). A selection medium (MSSr) is required to isolate transgenic callus expressing the *hpt* marker gene, and this is MSHr supplemented with 30 mg/L hygromycin B. The regeneration medium (MSRr) is the basic MS salts and organics recipe supplemented with 0.2 M maltose, 4 g/L gellan gum, 5 mg/L NAA, and 3 mg/L 6-BAP to encourage the growth of shoots and 30 mg/L hygromycin B

to prevent the emergence of “escapes” (nontransgenic cells that survive under selection). Maltose has been shown to promote green tissue growth better than sucrose and other carbon sources in a number of plant tissue culture systems (17–19), and we have found this also applies to callus derived from mature rice embryos. The rooting medium is similar to MSRR but replaces maltose with 10 g/L sucrose and contains no phytohormones.

7. We used five different endosperm-specific promoters in our pilot system (1), but we have found that using the same promoter for each transgene is just as effective (20).
8. Only high-quality supercoiled plasmid DNA should be used. We find that QIAGEN Maxiprep plasmid kits are satisfactory for this purpose. The eluted plasmid DNA should be precipitated with ethanol and redissolved in water to a concentration of 10 mg/mL. Check the concentration and purity using a Nanodrop 1000 UV spectrophotometer.
9. The selectable marker gene is provided on a separate plasmid. All transgenic plants carry this marker even though the recovery of other transgene combinations is random. This does not mean the marker is any more likely to integrate but reflects the fact that transgenic plants are propagated under selection, so only those carrying the marker survive.
10. A Bio-Rad pneumatic helium gun, model PDS 1000/He, is described here, but other devices can be used including homemade ones. When considering investing in or hiring a PDS 1000/He particle gun, the manufacturers will advise on setting up and operating procedures. Bottled helium gas, carrier sheets, rupture discs, and stopping sheets are available from the same supplier.
11. Tungsten particles are less expensive than gold ones, but we find gold particles more efficient for transformation, possibly because tungsten particles are less uniform in size and are more reactive than gold, resulting in more damage to the target tissue (reviewed in ref. 21). The size of the particles also influences transformation efficiency. Larger particles are generally faster and penetrate further but cause more damage. For rice and corn transformation, we recommend particles 0.7–0.9 μm in diameter.
12. The use of expensive covers for the culture vessels is not necessary. Aluminum foil is easily costerilized and provides a satisfactory safety level against contamination.
13. The optimal age of immature corn embryos for transformation is 14 days. The maturity of the embryos can be determined by their size (immature embryos will be 0.8–1 mm in length).
14. Osmoticum treatment has been shown to improve the efficiency of particle bombardment. The exact basis is unclear.

It may remove water droplets from the seed surface that deflect the metal particles or it may change the physiology of the cell (e.g., plasmolysis), making it more amenable to transformation (22). We use mannitol for this purpose in both corn and rice, although the corn protocol also includes sorbitol as this has been shown to stimulate the growth of embryogenic callus (23).

15. The optimal pressure and distance between the gun and the target needs to be determined empirically for each type of explant. For optimization, a transient expression assay is useful. Bombard the explant with a DNA construct comprising the visible marker *gusA* driven by a strong and constitutive promoter such as corn *Ubi-1*. Optimal transformation occurs when many small spots of GUS activity are detected 1–2 days after bombardment. If there are large sectors of GUS-positive tissue, this usually means the particle velocity is too high and the tissue is dead. Reduce the pressure or increase the distance to the target and try again.
16. The maturity of rice embryos can be estimated by pressing the seeds between the fingers. Immature seeds leave a milky liquid residue on the fingers whereas mature seeds do not. Callus derived from mature seeds is more efficient for transformation, but mature seeds are tougher and need to be dehusked with sandpaper.
17. Handle the dehusked seeds very carefully from this stage onward and use very gentle agitation.
18. Here, the aim is to preserve any embryogenic callus produced within the scutellar region of the mature embryos. Dead callus, which can be recognized by its brown or black colour, should be discarded at each subculture to prevent the proliferation of escapes.
19. We use a 4:2:1 mix of topsoil, sand, and pouzzelane (EuroPouzzelane, Lattes, France).
20. The amount depends on the colour intensity of the samples. For pale samples, extract 100 mg. For darker samples, it is better to extract 50 mg.

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Production of a His-Tagged Canecystatin in Transgenic Sugarcane

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Abstract

Transgenic plants have been widely used as expression systems of recombinant proteins in recent years because it can be an efficient alternative for the large-scale production of proteins. This is an area with great potential but is still not much explored. Indeed, this system can bring a breakthrough in the expression of any protein. The model used here as a protein factory was sugarcane, a crop of great global importance. This chapter describes the system that has been adopted in the routine production of transgenic sugarcane coupled with protein purification protocol. In this chapter, we describe production of transgenic sugarcane expressing a His-tagged cystatin under the control of the maize ubiquitin promoter. A transformed sugarcane plant presented high levels of protein expression and was selected for the purification of this protein through affinity chromatography in a nickel column. These studies demonstrate that sugarcane can be a viable expression system for recombinant protein production and that the His-tag purification strategy used to isolate the purified protein was effective.

Key words: Cystatin, Recombinant protein, Expression system, Transgenic sugarcane

1. Introduction

Protein expression in transgenic plants has been successful because plants can provide a cheap source of recombinant proteins, such as enzymes used in the pharmaceutical and cosmetic industries (1–5). Using the available structure for plant cultivation, it is possible to decrease the amount of capital investment required for commercial production of practically any protein. A number of factors have contributed toward the use of plants for the production of heterologous proteins. Several studies have been carried out confirming that the system is simple and commercially viable (6).

Apart from the economic advantages, there are qualitative benefits favoring the use of transgenic plants as factories for producing recombinant proteins (7, 8). The major advantage of transgenic plants over production systems is their ability to perform most of the posttranslational modifications that are required for the activity of some recombinant proteins. Furthermore, products from transgenic plants are unlikely to be contaminated by animal pathogens, microbial toxins, or oncogenic sequences (7).

In recent years, plant production systems have been developed for monoclonal antibody production, which has been useful in passive immunization of viral or bacterial diseases (9). The protein expression in transgenic plants is a well-established technology, and there are many recent reviews on this subject (7–10).

Sugarcane, which is one of the world's major crops, plays an especially important role in the Brazilian economy and is used for the production of sugar and alcohol, a renewable fuel that partially replaces oil by-products (11).

The cultivation of sugarcane in Brazil covers a large area, producing a huge amount of leaves that are likely to be discarded. In a short period, sugarcane cultivation produces a high green mass, energy, and fibers. At harvest, the leaves are discarded or burned. Therefore, taking advantage of this fact, the leaves can be collected and utilized for the extraction of recombinant proteins. Moreover, the large-scale expression of proteins from discarded residue and subsequent commercialization of this technology will provide greater and cheaper access to products.

The protein chosen to validate the recombinant expression system in sugarcane was a cystatin. Cystatin is a reversible inhibitor of cysteine peptidase, and their mode of action is based on competitive inhibition through the blocking of proteolytic activity (12). The use of protease inhibitor proteins in the development of pest-resistant transgenic plants is an important strategy that has been adopted in recent years (13). The ingestion of protease inhibitors by pathogens interferes in the protein degradation process, which reduces the availability of amino acids, thereby hindering the synthesis of proteins necessary for the growth, development, and reproduction of the pathogen (14).

Therefore, an interesting alternative would be to develop transgenic sugarcane expressing cystatins. This procedure can offer benefits to agriculture, including the increased resistance of the plant against pests as well as the use of this transgenic plant as an expression system for producing recombinant proteins on a large scale.

In this chapter, we present the transformation of sugarcane with a His-tagged canacystatin (CaneCPI-1) (15) construction in order to test the wider viability of using transgenic sugarcane plants as a source for the production of recombinant proteins.

2. Materials

2.1. Plant Material, Plasmids, and Bacterial Strains

1. Sugarcane variety SP80-185 (*Saccharum officinarum* L.).
2. *Escherichia coli* strain used was DH5- α .
3. Plasmid constructs pAHC17 (16) and pHA9 (17).

2.2. Enzymes and Commercial Kits

1. *Bam* HI (Invitrogen, Carlsbad, CA, USA).
2. *SAP—Shrimp alkaline phosphatase enzyme* (Promega, Madison, WI, USA).
3. T4 DNA ligase (Invitrogen, Carlsbad, CA, USA).
4. QIAprep Miniprep Kit (Qiagen, Valencia, CA, USA).
5. QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).
6. Plant DNAzol (Invitrogen, Carlsbad, CA, USA).
7. *Taq DNA Polymerase* (Invitrogen, Carlsbad, CA, USA).
8. Petri dishes (9-cm diameter).

2.3. Growth Media, Reagents, and Special Laboratory Equipment for Sugarcane Transformation

All media, buffers, and other solutions are made up as aqueous preparations. The media for tissue culture are agar-solidified in 9-cm Petri dishes (25 mL/Petri) after sterilization using standard procedures.

1. Cl-3 induction medium: (MS) medium (18) supplemented with 5% coconut water, 2,4-dichlorophenoxyacetic acid (2,4-D) 3 mg/L, antibiotic Clavulin (Beecham, Brentford, Middlesex, United Kingdom) 0.5 g/L solidified with 8 g/L of agar after adjusting the pH to 5.8 (19) (see Note 1).
2. Cl-3 regeneration medium: (MS) medium (18) supplemented with 5% coconut water, BAP (6-Benzyladenine), 35 mg/L antibiotic Geneticin (Invitrogen, Carlsbad, CA, USA) solidified with 8 g/L of agar after adjusting the pH to 5.8 (see Note 2).
3. A tissue-culture room, maintained at 25°C, and a photoperiod of 12 h/day provided by cool white fluorescent lights.
4. A controlled-environment chamber, maintained at 20°C (day) and 15°C (night), a relative humidity of 75–80%, and a photoperiod of 12 h/day provided by cool white fluorescent lights.
5. Liquid nitrogen and a mortar and a pestle.
6. Biolistic transformation equipment.
7. Tungsten particles M20 1.32 μm (Bio-Rad, Hercules, CA, USA), sterilized in 70% ethanol, as a 60 mg/mL suspension in sterile UHP water.
8. 2.5 M CaCl_2 in UHP water, sterilized by autoclaving.

9. Geneticin (Invitrogen, Carlsbad, CA, USA) stock solution of 25 mg/mL, filter-sterilized.
10. Spermidine (Invitrogen, Carlsbad, CA, USA).

2.4. Purification of Protein from Transgenic Plant: Buffers and Reagents

1. Protein extraction buffer: Tris-HCl 0.2 M containing 32 mM Na_2HPO_4 , 84 mM citric acid, and 0.1% β -mercaptoethanol, pH 8.0.
2. Lysis buffer: 10 mM Tris-HCl, 50 mM NaH_2PO_4 , and 50 mM NaCl, pH 8.0.
3. Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA).
4. Nickel-immobilized resin Ni-NTA agarose superflow (Qiagen).

2.5. Electrophoretic Methods

2.5.1. SDS-Polyacrylamide Gel Electrophoresis Buffers, Reagents, and Equipment

1. Running gel: 10% (w/v) polyacrylamide (a mixture of 97% acrylamide and 3% bisacrylamide) in 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate, and *N,N,N',N'*-tetramethylene diamine (TEMED).
2. Upper gel: 5% (w/v) polyacrylamide (*acryl-bis-acrylamide* mixture) in 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.03% (w/v), and TEMED.
3. Running buffer: 25 mM Tris-HCl, 192 mM glycine, and 0.01% (w/v) SDS, pH 8.3.
4. SDS sample buffer: 367 mM Tris-HCl, pH 6.8, 30% (v/v) mercaptoethanol, 30% (v/v) glycerol, 0.003% bromophenol blue, and 4% (w/v) SDS.
5. *BenchMark* Protein Ladder (Invitrogen, Carlsbad, CA, USA) for use as molecular weight standards for SDS-polyacrylamide gel electrophoresis (range of 10–220 kDa).
6. Mini-Protean II TM slab-gel unit (Bio-Rad, Hercules, CA, USA).

2.5.2. Western Blotting

1. Blotting buffer: 25 mM Tris-HCl, 192 mM glycine, and 10% (v/v) methanol, pH 8.3.
2. Invitrolon PVDF membrane filters (Invitrogen, Carlsbad, CA, USA) and 3MM chromatography paper (Whatman, Maidstone, UK).
3. Ponceau S (Sigma, St. Louis, MO, USA) in 3% (w/v) TCA.
4. Tris-buffered saline (TBS): prepare 10 $\times$ stock TBS buffer: 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Dilute 100 mL with 900 mL water for use.
5. Blocking buffer: 1% (w/v) powdered skim milk in TBS buffer.

6. Primary antibody: polyclonal mouse antiserum against CaneCPI-1 diluted 1:10,000 in blocking buffer.
7. Primary antibody: anti-His antibody (GE Healthcare, Chalfont St Giles, UK) diluted 1:10,000 in blocking buffer.
8. Secondary antibody: anti-mouse IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA) diluted 1:10,000 in TBS buffer.
9. Staining reagent AP Conjugate Substrate Kit (Bio-Rad, Hercules, CA, USA).

2.5.3. Activity Assay of Purified Recombinant Cystatin

1. Fluorogenic substrate Z-Phe-Arg-MCA (Calbiochem, CA, USA).
2. Hitachi F-2000 Spectrofluorometer.
3. Cathepsin L (Calbiochem, CA, USA).
4. 100 mM sodium acetate buffer, pH 5.5.
5. 1.0 mM DTE—dithioerythritol (Calbiochem, CA, USA).
6. Quartz cuvette (1 mL).
7. Canecystatin recombinant protein purified from transgenic sugarcane.

3. Methods

This section describes simple procedures and strategies for obtaining transgenic sugarcane plants expressing CaneCPI-1, for assessing the recombinant inhibitor expressed in leaf tissues, and for achieving its efficient recovery from the plant tissues with a view to using transgenic sugarcane as system for the production of recombinant proteins.

Provided that efficient transformation procedures are available, the approaches presented to analyze and to recover recombinant CaneCPI-1 may be used for any protein, without the need for major modifications. The methods described below outlines construction of the expression plasmids, plant transformation, and analysis of protein expression in the sugarcane transgenic plants and purifications of CaneCP-1 recombinant protein.

3.1. Construction of Expression Plasmid

The expression vector used in sugarcane plants is pAHC17 (16), as it possesses the maize ubiquitin promoter (*Ubi-1*) (see Note 3).

The construction is denominated pAHCaneCPI-1C (20); it possesses the cDNA coding the cysteine peptidase inhibitor protein (CPI) of sugarcane (Accession Number. AY119689), originated from the Sugarcane Genome Project-SUCEST (FAPESP) cloned in fusion with His-tagged histidine in C-terminal region. This His-tag

Fig. 1. Schematic representation of CaneCPI-1 expression vector pAHCaneCPI-1C. *Ubi-1* promoter, maize ubiquitin promoter; CaneCPI-1 ORF; 6× Histidine tag; NOS (nopaline synthase terminator). The *arrow* indicates the ATG initiation codon of CaneCPI-1 protein, the stop codon is *underlined*, and the restriction enzyme site for *Bam* HI enzyme is in *bold*. Reproduced from ref. 20 with permission from ACS Publications Division Permissions, John Wiley and Sons.

The pieces of stem (30 cm long) are obtained from sugarcane variety SP80-185 (*Saccharum officinarum* L.) and subjected to heat shock at 55°C for 15 min, and then washed with water and planted in cell foam containing the Plantimax substrate. The plants are

maintained for 3 months in a greenhouse until they reach 3 m in length. To obtain the meristem region, take following steps:

1. Collect the apical region of the stem of the sugarcane (*S. officinarum* L.) cultivar SP80-185 from 10-month-old field-grown plants (see Note 4).
2. Remove the older leaves and a 30-cm-long leaf roll containing the apical meristem and disinfect with a 2% sodium hypochlorite solution for 15 min.
3. Excise transverse segments (3 mm) of the leaf roll from within 10 cm above the apical meristem (21).

3.2.2. In Vitro Culture

1. To initiate the induction of embryogenic calli from transverse segments of immature leaves on induction CI-3 medium, supplement the medium with 5% coconut water, 13.5 mM 2,4-dichlorophenoxyacetic acid (2,4-D), and Clavulin antibiotic and solidify with 8 g/L of agar after adjusting the pH to 5.8 (see Note 5).
2. Cultivate the explants in the dark at 25°C for 30 days.
3. Subculture calli and transfer to fresh medium under the same conditions.
4. Select embryogenic calli after 30 days, under a dissecting microscope based on morphological characteristics (nodular, compact, and white calli), and then subculture for another 25 days under the same conditions (see Note 6).
5. Four hours before bombardment, put the calli on the circle (3-cm diameter) on the same culture medium, supplemented with 0.4 M mannitol and 0.4 M sorbitol as an osmotic treatment modified from ref. (22) (see Note 7).

3.2.3. Bombardment

The bombardment equipment used was developed by the Center for Sugarcane Technology (CTC) and employs compressed gas helium 9 kgf/cm², and the shots are made in a vacuum chamber of 26 mmHg to minimize air resistance (23).

1. Co-bombardment of sugarcane calli should be initiated with plasmid vector pAHCaneCPI-1C together with pH A9 (17), which carry the geneticin resistance gene (24) (see Note 8).
2. Co-precipitate the plasmids at rate of 5 µg of each plasmid DNA (1 µg/µL) for each tungsten particle (average size 1.2 µm) in a similar method to that described by Birch and Franks (25).
3. Mix the tungsten particles in a suspension of 50% glycerol (25 µL) with 5 µL of each plasmid DNA (1 µg/µL), 25 µL of CaCl₂ (2.5 M), and 10 µL of spermidine (0.1 M, free base form) in a microcentrifuge tube at 4°C.

4. Incubate the mixture for 15 min on ice and centrifuge briefly.
5. Remove 50 μ L of the supernatant; resuspend the tungsten pellet in the remaining solution.
6. Use 2 μ L of the particle suspension for bombardment.
7. Place calli in Petri dishes of 14 cm away from the syringe filter unit.
8. Place a nylon mesh screen (500 μ m) on top of the Petri dish to reduce calli movement when shooting.
9. Perform bombardment under vacuum using a helium pressure of 784.5 kPa.
10. Shoot the vacuum pump by pressing a button.
11. Release vacuum immediately after bombardment.

3.2.4. Regeneration and Selection of Transformed Plants

The regeneration of shoots from embryogenic calli is performed at 25°C under a 12-h photoperiod with 10 calli/Petri dish (see Note 9). The steps of regeneration are as follows:

1. After the bombardment, transfer the calli to Cl-3 medium plus 2,4 D (3 mg/L) and maintain in the dark for 10 days.
2. After 10 days, transfer the calli to Cl-3 medium containing 2,4 D and the antibiotic geneticin (35 mg/L) and maintain at photoperiod of 12-h light/12-h dark at 25°C for selection of transformants over a 6-week period.
3. Place resistant calli on the Cl-3 medium to regenerate plants over approximately 3 months for development of plants with roots (see Note 10).

The plantlets should be maintained in a culture room at 25°C with a 12-h photoperiod until they reach 4 cm.

3.2.5. Second Stage of Plant Selection Using PCR

In the second stage of selection, it is possible to analyze transgenic plants to verify vector insertion through PCR and to determine the integration of the His-tagged canecystatin gene in the sugarcane genome.

The amplification reaction is accomplished using the following primers: Ubi_forward: 5' GGCATATGCAGCATCTATTCA 3' and CaneR_bam: 5' CAGCAGGATCCTTAGGCGTCCCCGAC CGGCTG 3', which hybridizes in the ubiquitin promoter and 3' flanking region of the canecystatin gene, respectively. The thermocycler program is [1 $\times$] 94°C 1 min, [40 $\times$] 94°C 30 s, 48°C 30 s, 72°C 1 min, and 72°C 5 min. An example of this type of result is shown in Fig. 2.

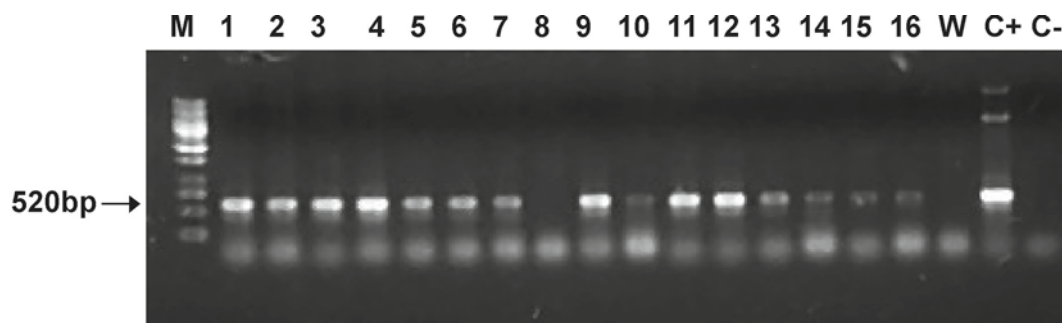


Fig. 2. Amplified products of DNA samples from sugarcane leaves of transformed plants, pAHCaneCPI-1C construction. Electrophoretic agarose gel showing M: 1-kb ladder; lines 1–16: selected plants, W: non-transformed plant, C+ positive control (pAHCaneCPI-1C), C– negative control. Reproduced from ref. 20 with permission from ACS Publications Division Permissions, John Wiley and Sons.

3.3. Purification of Recombinant CaneCPI-1

3.3.1. Preparation of Samples for Purification of Recombinant ^{HIS}CaneCPI-1 by Affinity Chromatography

The protocol of protein extraction is based on method described in ref. (26) with modifications.

1. Weigh approximately 10 g of sugarcane leaves and grind into a powder in liquid nitrogen using mortar and pestle.
2. Homogenize the powder in 30 mL of extraction buffer (Tris 0.2 M containing 32 mM Na_2HPO_4 , 84 mM citric acid, and 0.1% β -mercaptoethanol, pH 8.0 with 1 mM HCl) in a 2:1 proportion (mL:g tissue fresh weight) (see Note 11).
3. Shake the homogenate for 45 min, filter through four gauze layers, and centrifuge at $10,000 \times g$ for 30 min.
4. Add to the supernatant an equal volume of 10% trichloroacetic acid, incubate for 1 h, and centrifuge at $10,000 \times g$ for 30 min.
5. Resuspend the precipitate in 5 mL of cold trichloroacetic acid, shake, and centrifuge again under the same conditions.
6. Wash three times with ethanol and leave to dry overnight.
7. Resuspend the precipitate in 10 mL lysis buffer containing 10 mM Tris–HCl, 50 mM NaH_2PO_4 , and 50 mM NaCl, pH 8.0.
8. The recombinant protein is purified from the crude extract of sugarcane leaves using a nickel affinity resin column previously equilibrated with five column volumes of lysis buffer containing 10 mM Tris–HCl, 50 mM NaH_2PO_4 , and 50 mM NaCl, pH 8.0.

9. Add the sample to the column and wash with three column volumes of lysis buffer.
10. Recombinant protein is eluted with one volume of lysis buffer containing increasing concentrations of imidazole (10, 25, 50, 75, 100, and 250 mM).
11. After protein elution, make a dialysis in a buffer containing 10 mM Tris-HCl and 50 mM NaCl, pH 8.0.
12. Purified products are analyzed in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 15% (27), and protein concentration is determined using the Bradford method (28).

3.3.2. Western Blotting for Canecystatin Protein

The Western blotting protocol was performed according to ref. (29).

1. For the Western blotting analyses, separate equal amounts of protein by SDS-PAGE 15% (27) and transfer to Invitrolon PVDF membrane filters (Invitrogen).
2. Filters are blocked with 5% defatted milk in TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) (see Note12).
3. Wash the membrane four times in TBS buffer and probe with specific antibodies anti-CaneCPI-1 or anti-His-tag at dilutions of 1:5,000 and 1:10,000, respectively.
4. Incubate the membrane with antibody AP-labeled anti-mouse IgG antibody (Sigma) at dilution of 1:10,000. Antibody reacting protein bands are revealed using the AP Conjugate Substrate Kit (Bio-Rad). An example of the type of result is shown in Fig. 3.

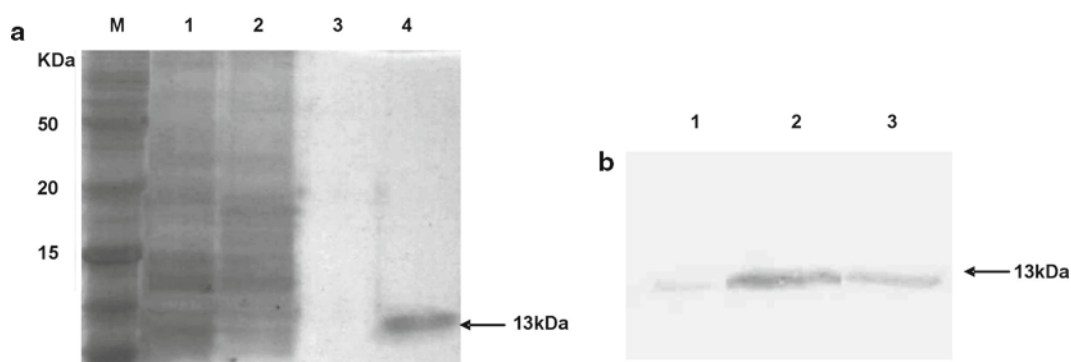


Fig. 3. Analysis of purification and immunodetection of the $_{\text{His}}$ CaneCPI-1 in transgenic sugarcane. (a) $_{\text{His}}$ CaneCPI-1 purification: SDS-PAGE (15%) stained with coomassie blue. In M: molecular mass marker (Invitrogen); in 1: crude extract of transformed sugarcane leaves; in 2: crude extract of non-transformed sugarcane leaves; in 4: eluted fraction in 250 mM of imidazole (to show a 14.7-kDa band). (b) Immunodetection with anti-CaneCPI-1 of the crude extract of non-transformed and transformed sugarcane. In 1: purified $_{\text{His}}$ CaneCPI-1 of transformed sugarcane; in 2: crude extract of transformed sugarcane; and in 3: crude extract of non-transformed sugarcane.

3.3.3. Inhibitory Activity of Purified Canecystatin Protein

To verify the inhibitory activity of purified canecystatin recombinant protein, a test of cysteine proteinase inhibition is carried out. For activity test, a human cysteine peptidase cathepsin L (Calbiochem, CA, USA) is used in the presence of the fluorogenic substrate Z-Phe-Arg-MCA (Calbiochem, CA, USA). The activity of cysteine peptidase is determined spectrofluorometrically, based on the procedure described by Anastasi et al. (30). To assay, use a Hitachi F-2000 Spectrofluorometer at excitation and emission wavelengths of 380 and 460 nm, respectively. In a quartz cuvette (1 mL working volume), add the following reaction components:

1. 500 μ L of 100 mM sodium acetate buffer, pH 5.5.
2. Wait 30 min for heating equipment at 37°C (see Note 13).
3. Add cathepsin L enzyme in the cuvette at a final concentration of 0.3 nM.
4. Pre-activate the enzyme in the presence of 1.0 mM DTE (dithioerythritol) for 5 min at 37°C, before the addition of the substrate.
5. Add 1 μ L of purified canecystatin protein at a final concentration of 1 nM.
6. Add fluorogenic substrate Z-Phe-Arg-MCA at the final concentration of 0.01 mM.
7. Measure fluorescence released by hydrolysis of the substrate Z-Phe-Arg-MCA by cathepsin L in Hitachi F-2000 Spectrofluorometer in presence of inhibitor.
8. Inhibitory activity of canecystatin is determined by measuring the residual hydrolytic activity of cathepsin L.
9. Repeat steps 6–8 with increasing concentration of inhibitor (canecystatin).
10. Plot a graph containing increasing inhibitor concentrations (canecystatin) by enzymatic activity of cathepsin L.
11. To determine if the inhibitor canecystatin is active, calculate the inhibition constant value (K_i).
12. The inhibition constant (K_i) of the enzyme-inhibitor complexes is calculated following Morison's procedure (31).

4. Notes

1. Clavulin antibiotic is manufactured by SmithKline Beecham Pharmaceuticals; the generic name is amoxicillin and potassium clavulanate. The presence of this type of antibiotic is important to avoid the contamination in the culture medium.

2. The plant growth regulator BAP (6-benzylaminopurine) is a cytokinin. These are a large group of structurally related purine derivatives that promote cell division.
3. The pAHC17 plasmid (16) has a ubiquitin promoter, which has been shown to be significantly more active in monocot cells.
4. Apical meristematic regions that contain young-curved, protected leaves are taken from 3-month-old plants (30 cm in length) grown in a green house.
5. The regeneration response was evaluated in each culture cycle by scoring regenerants with only shoots, only roots, or shoots and roots. When choosing a selection agent, it is important to test for natural resistance of the target plant material by first performing a kill curve experiment and establishing the optimum concentration.
6. Plant material may exhibit two types of calli: one friable and translucent and one with nodular domes, not friable, and usually embryogenic.
7. After 19 weeks, the calli were selected and transferred to medium containing sorbitol and mannitol (30 g/L) 4 h before the bombardment. The Cl-3 medium containing sorbitol and mannitol increases osmolarity of the cell causing plasmolysis which facilitates the penetration of tungsten particles with no degradation of cell wall and cell membrane.
8. The pAH9 plasmid contains the *neo* gene, placed under the control of the maize ubiquitin promoter, *ubi-1*, with the nopaline synthase 3'end.
9. The regeneration of shoots from embryogenic calli is performed after several processes of selection based on appearance of the calli. The calli with a rusty appearance are discarded, and normal calli are selected for the next phase.
10. To obtain control (non-transformed plants) through regeneration from the same callus type, undergo the same tissue culture steps without bombardment.
11. At the moment of adding of 10% TCA, protein precipitation will occur. After centrifugation, discard the supernatant and be careful to avoid loss of the protein. The protein has a gelatinous aspect because of the amount of polysaccharides from sugarcane, and its tendency not to adhere to the tube wall can lead to the loss of the protein at this stage.
12. After blocking of the membrane filter, it is possible to store the PVDF membrane for a period of 7 days. First, remove excess defatted milk, wash membrane with water, and place in a freezer at -20°C .

13. The heating of equipment is essential for activity of the enzyme assay. The target temperature should be the same as that for enzyme activity, which can vary between 30°C and 40°C depending on the enzyme to be used in the test.

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Plastid Transformation as an Expression Tool for Plant-Derived Biopharmaceuticals

Nunzia Scotti and Teodoro Cardi

Abstract

The production of biopharmaceuticals in plants is currently one of the most attractive approaches to modern medicine. Several efficient plant-based expression systems have been developed so far. Among them, plastid transformation has attracted biotechnologists because the plastid genome, unlike nuclear genome, bears a number of unique advantages for plant genetic engineering. These include higher levels of protein production, uniform gene expression of transformants due to the lack of epigenetic interference, and expression of multiple genes (as in operons) from the same construct. Further, the plastid transformation technology is an environmentally friendly method because plastid and their genetic information are maternally inherited in many species with a consequent lack of transmission of plastid DNA by pollen. Recently, great progress has been made with plastid-based production of biopharmaceuticals demonstrating that it is a promising platform for such purposes. This chapter describes detailed protocols for plastid transformation including the delivery of DNA by biolistic method, the selection/regeneration of transplastomic plants, and the molecular analyses to select homoplasmic plants and confirm transgene expression.

Key words: Plastid transformation, Tobacco, Biofactories, Biopharmaceuticals, Transgene containment

1. Introduction

Plastids are a family of plant organelles derived from endosymbiosis of a common ancestor of the present cyanobacteria. In plant cells, they participate in a wide range of metabolic processes (1). The chloroplast, the best known among plastids, is the site of photosynthesis and a number of biosynthetic pathways, including those for fatty acids, amino acids, isoprenoids, etc. All plastids derive, directly or indirectly, from a small and undifferentiated organelle, termed the proplastid, and share a common small circular DNA molecule (plastome) of about 120–160 kb present as numerous copies in each plastid (up to 10,000 copies/cell).

In recent years, plastids have long been the subject of biotechnological approaches based on plastid transformation opening new opportunities in plant biotechnology. Plastid transformation has many advantages, over conventional transgenic plants, linked to a very high levels of gene expression (up to 70% of total soluble proteins (2)), ensured by the high copy number of plastomes per plastid/cell, absence of gene silencing mechanisms, expression of polycistronic mRNAs, and gene containment due to the maternal inheritance of plastids in most crop species (3–5).

Recently, the expansion of the molecular farming concept (production of biopharmaceuticals in plants) has given an extensive boost to the exploration of the suitability of chloroplast genetic engineering for such purposes. In fact, great and exciting progress has been made with plastid-based production of biopharmaceuticals.

Several proteins belonging to the different subclasses of biopharmaceuticals (vaccines, therapeutic proteins, autoantigens, antibiotics, etc.) have been produced by plastid transformation (6–8), demonstrating that plastid transformation is a promising tool for plant-derived pharmaceuticals.

To date, the species of choice for plastid transformation is tobacco because of its large biomass and suitability for efficient transformation and regeneration. In fact, the main limitation of this technology is currently its extension to plants other than tobacco due to low transformation efficiency. Generally, it has been attributed to several factors such as the kind of explants, the regeneration protocol, the nonoptimal homology of flanking regions etc. (9–14). In some species, the use of species-specific transformation vectors and improved regeneration protocol resulted in an improvement of the transformation efficiency (15–20). Despite the progress made for plants other than tobacco, only two crops (tomato and lettuce) have been used successfully for the production of biopharmaceuticals via plastid transformation (21–23). Another limitation of plastid transformation is the absence of some posttranslational modifications such as glycosylation important for biological activity of some biopharmaceuticals.

In this chapter, we describe detailed procedures (delivery of DNA by biolistic method, selection/regeneration of transplastomic plants, molecular analyses to select homoplasmic plants, and confirm transgene expression) for chloroplast transformation of tobacco used in our laboratory to produce plant-derived viral antigens (HIV, HPV, Vaccinia virus) and other recombinant proteins (24–26), with particular emphasis on plant-derived HIV-1 Pr55^{gag} polyprotein.

2. Materials

2.1. Particle Bombardment

1. PDS-1000/He™ Gene Gun delivery system (Bio-Rad, Hercules, CA, USA), gold particles (microcarrier, 0.6 µm), macrocarriers, stopping screen, and rupture disks (1,100 psi).
2. *Nicotiana tabacum* (in vitro-grown plants).
3. 2.5 M CaCl₂ (sterilize by filtration, should always be prepared fresh).
4. 0.1 M spermidine (prepare a stock, aliquot into small volume, and store at -20°C, see Note 1).
5. 70% and 100% ethanol (molecular grade).
6. Sterile water.

2.2. Tobacco Plant Tissue In Vitro Culture

1. RMOP medium: Murashige and Skoog salts 4.30 g/L, thiamine 1 mg/L, *myo*-inositol 100 mg/L, sucrose 30 g/L, benzylaminopurine 1 mg/L, naphthylacetic acid 0.1 mg/L, and Micro Agar 8 g/L. Adjust pH to 5.8 before autoclaving medium (All chemicals from Duchefa, Haarlem, The Netherlands).
2. Sterile forceps and scalpel.
3. Sterile Petri dishes (100×20 mm and 60×15 mm).
4. Antibiotics: prepare stock solutions of spectinomycin (100 mg/mL) and streptomycin (250 mg/mL).
5. MS30B5 medium: Murashige and Skoog salts 4.30 g/L, B5 vitamins 1 mL/L, sucrose 30 g/L, and Micro Agar 8 g/L. Adjust pH to 5.8 before autoclaving medium (All chemicals from Duchefa).

2.3. Identification of Transplastomic Plants by PCR

1. Total DNA from transplastomic and untransformed (wild-type) plants.
2. PCR primers: primer 1 which anneals to the native integration plastid sequence and primer 2 which anneals to the gene of interest.
3. dNTPs.
4. Taq DNA polymerase, MgCl₂, and buffer (Invitrogen, Carlsbad, CA, USA).
5. DNA molecular weight marker (Fermentas, Ontario, Canada).
6. Agarose (Invitrogen).

2.4. Identification of Homoplasmic Plants by Southern Blot Analysis

1. Total DNA from transplastomic and untransformed (wild-type) plants.
2. Restriction enzymes and buffer (New England Biolabs, Ipswich, MA, USA, supplied by manufacturer).
3. DNA molecular weight marker (Fermentas).

4. Agarose (Invitrogen).
5. 0.25 N HCl (depurination solution).
6. 0.4 N NaOH (transfer buffer).
7. Nylon membrane (Hybond-N+, GE Healthcare, Waukesha, WI, USA).
8. GS GeneLinker UV Chamber (Bio-Rad).
9. Flanking probe produced by PCR amplification of the flanking sequences using digoxigenin-11 dUTP (Roche Applied Science, Penzberg, Germany) and two primers which anneal to the native plastid integration sequences (see Note 2).
10. Hybridization oven (Thermo, Waltham, MA, USA) and roller bottles.
11. DIG Easy Hyb Granules, blocking solution, anti-digoxigenin-AP conjugate, and CSPD (Roche Applied Science, DIG High Prime DNA labeling and Detection Starter Kit II).
12. 2× SSC, 0.1% SDS and 0.5× SSC, 0.1% SDS.
13. Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, and adjust pH to 7.5 with solid NaOH.
14. Washing buffer: 0.1 M maleic acid, 0.15 M NaCl, adjust pH to 7.5, and 0.3% (v/v) Tween 20.
15. Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, and adjust pH to 9.5.
16. Autoradiography cassette and film.

2.5. Protein Extraction and Western Blot Analysis to Evaluate the Expression of Recombinant Proteins

1. Eppendorf tube (1.5 mL) and polypropylene pestle.
2. Liquid nitrogen.
3. 10× PBS: 80 g/L NaCl, 2 g/L KCl, 14.4 g/L Na₂HPO₄, 2.4 g/L KH₂PO₄, and adjust pH to 7.5.
4. PBS HS: 1× PBS (1:10 diluted from 10× PBS), 0.5 M NaCl, 10 mM EDTA, 1 mM PMSF, and 5 mM DTT.
5. Bradford assay (Bio-Rad protein assay).
6. Loading sample buffer (6×): 50 mM Tris-HCl pH 6.8, 2% SDS, 2 mM EDTA, 5 mM DTT, 10% glycerol, and 0.1% blue bromophenol.
7. 10% SDS and 100 mM PMSF (see Note 3).
8. Acrylamide/bis (ready to use mixture from Bio-Rad).
9. TEMED (ready to use, Bio-Rad).
10. Separating buffer (4×): 1.5 M Tris-HCl pH 8.8 and 0.4% SDS.
11. Stacking buffer (4×): 1 M Tris-HCl pH 6.8 and 0.4% SDS.
12. 10% Ammonium persulfate (should always be prepared fresh).
13. Running buffer (5×): 25 mM Tris-HCl pH 8.8, 250 mM Glycine, and 0.5% SDS.

14. Prestained molecular weight marker (Fermentas).
15. Blotting buffer (10×): 48 mM Tris and 39 mM glycine.
16. 1× Blotting buffer (1:10 diluted from 10× blotting buffer) and 10% (v/v) methanol.
17. Nitrocellulose membrane (GE Healthcare).
18. 3MM chromatography paper (Whatman, Maidstone, UK).
19. Bovine serum albumin (BSA, Sigma, St. Louis, MO, USA).
20. Nonfat dry milk (Nestlé Carnation, Vevey, Switzerland).
21. Blocking buffer: 1× PBS and 3% BSA.
22. T-PBSW: 1× PBS containing 0.1% Tween 20.
23. Antibodies: antigen-specific primary antibody (ARP432 and ARP431, Programme EVA Centre for AIDS Reagents, NIBSC, UK); secondary antibody conjugated to horse radish peroxidase (peroxidase-labeled anti-rabbit antibody, GE Healthcare).
24. Enhanced chemiluminescent reagents (ECL plus Western blotting detection system, GE Healthcare).
25. ECL film (GE Healthcare).

**2.6. Enzyme-Linked
Immunosorbent Assay
to Quantify the
Expression Level of
Recombinant Proteins**

1. EIA-RIA 96-well plate.
2. 1× PBS.
3. T-PBSE: 1× PBS containing 0.05% Tween 20.
4. T-PBSEM5: T-PBS containing 5% nonfat dry milk.
5. T-PBSEM1: T-PBS containing 1% nonfat dry milk.
6. Antigen standard: HIV-1 p24 protein purified from *E. coli* (EVA 673, Programme EVA Centre for AIDS Reagents, NIBSC, UK).
7. Antigen-specific primary antibody: mouse monoclonal antibody anti-p24 (ARP 3243.4, Programme EVA Centre for AIDS Reagents, NIBSC, UK); rabbit polyclonal antibody anti-p24 (ARP432, Programme EVA Centre for AIDS Reagents, NIBSC, UK).
8. Secondary antibody: horseradish peroxidase (HRP)—conjugated anti-rabbit antibody (GE Healthcare).
9. TMB substrate (Bio-Rad).
10. 2 N Sulfuric acid.
11. Microtiter plate reader (Victor, Perkin Elmer, Waltham, MA, USA).

**2.7. Extraction
of Chloroplast
Proteins by
Differential
Centrifugation**

1. Homogenization buffer: 50 mM Tris-HCl pH 8.0, 1.3 M NaCl, 25 mM EDTA pH 8.0, 0.2% BSA, 0.05% cysteine, and 56 mM β -mercaptoethanol.
2. Lysis buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5 mM DTT, 1 mM PMSF, and 0.5% Triton X-100.

3. Miracloth Calbiochem (Merck, Darmstadt, Germany).
4. Bradford assay (Bio-Rad protein assay).

3. Methods

In this chapter, we describe the biolistic DNA delivery method to transform plant plastids. It is a system of choice for most laboratories and requires a PDS-1000/HeTM Gene Gun delivery apparatus. DNA from plastid transformation vector is coated on gold particles, delivered at high velocity to go through the cell wall and membranes (both cellular and plastidial), then integrated into the plastome by a double recombination event. Further, detailed protocols for in vitro culture to select and regenerate transformants, and to confirm transformation and transgene expression are reported.

3.1. Preparation of Gold Particle Stock and Coating with DNA

1. Take 35 mg of gold microcarrier (0.6 μm) in an Eppendorf tube, add 1 mL of 100% ethanol, and vortex for 5 min.
2. Spin for 2 s at $4,300\times g$.
3. Carefully remove the supernatant, add 1 mL of 100% ethanol, and vortex for 5 min (repeat steps 2 and 3 three times).
4. To perform five shots, transfer 50 μL of microcarrier stock in a new Eppendorf tube and spin for 2 s at $4,300\times g$, remove the supernatant, and wash with 50 μL of cold sterile water (repeat three times).
5. Add sequentially 10 μL of plasmid DNA (1 $\mu\text{g}/\mu\text{L}$), 50 μL 2.5 M CaCl_2 , and 20 μL 0.1 M spermidine and vortex the mixture for 20 min at 4°C .
6. Precipitate the plasmid DNA on microcarrier gold, add 200 μL of cold ethanol to the mixture, and spin for 2 s at $4,300\times g$.
7. Remove carefully the supernatant, wash the pellet by adding 200 μL of cold ethanol, and spin for 2 s at $4,300\times g$ (repeat four times).
8. Resuspend the DNA-coated gold particles in 30 μL of ethanol and store on ice.
9. Before shooting, spread 5 μL of particles over the center of the macrocarrier, and as soon as they are dry, they are ready for use in biolistic transformation.

3.2. Particle Delivery into Tobacco Leaf Tissue

1. Harvest leaves from 20 to 30-day-old in vitro-grown tobacco plants.
2. Place the leaf on RMOP medium (without antibiotics) in Petri dish (60 $\times$ 15 mm) with the abaxial side uppermost.
3. Switch on the vacuum pump.

4. Open the knob of the helium gas and set up the helium pressure 200 psi higher than desired pressure of the rupture disk.
5. Switch on the PDS system.
6. Place the rupture disk in its holder ring and screw the holder into the PDS chamber.
7. Place the stopping screen in the retaining assembly. On top of this place, upside down the macrocarrier with DNA on it and screw the metal ring.
8. Place the retaining assembly in the fifth slot from the bottom in the PDS chamber.
9. Place the Petri dish containing the tobacco leaf into the target plate holder, then into the third slot (6 cm) from the bottom of the PDS chamber, and close the chamber door.
10. Turn the vacuum button on VAC position. Allow the vacuum pressure to reach 27–28 in. of Hg, turn the vacuum button on HOLD position, and press continuously the FIRE (shooting) button until the rupture disk bursts.
11. Release the FIRE button and turn the vacuum button to VENT position. Remove the bombarded sample when the vacuum pressure reaches 0 in. of Hg.
12. Repeat steps 6–11 for additional samples.
13. Shut down the system by rotating the knob of the gas cylinder clockwise. Create vacuum as in step 10 to release the gas pressure remaining inside the helium tube.
14. Press and release the FIRE button until the pressure falls to zero on the meter gauge of the gas cylinder.
15. Switch off the vacuum pump and PDS system.
16. Place the Petri dishes containing the bombarded leaves in the culture room for 2 days.

3.3. In Vitro Regeneration and Selection of Shoots

1. Cut each bombarded leaf into small pieces (3×3 mm) and place them (see Note 4) with their abaxial side uppermost on RMOP selection medium containing 500 mg/L of spectinomycin for the first round of selection in the growth chamber under dim light (about $10 \mu\text{E}/\text{m}^2/\text{s}$), in 16-h light, and 8-h dark regime. Within 4–6 weeks, putative transformed shoots should appear (Fig. 1).
2. Cut each primary shoot into small pieces (3×3 mm) and place two-third of them on RMOP selection medium containing 500 mg/L of spectinomycin (second round of regeneration) and one-third of them on RMOP selection medium containing 500 mg/L of both spectinomycin and streptomycin. If the primary shoot is a true transformant, the tissue will form a green callus on Spec-Strep plate (Fig. 2) and secondary green shoots on Spec plate.

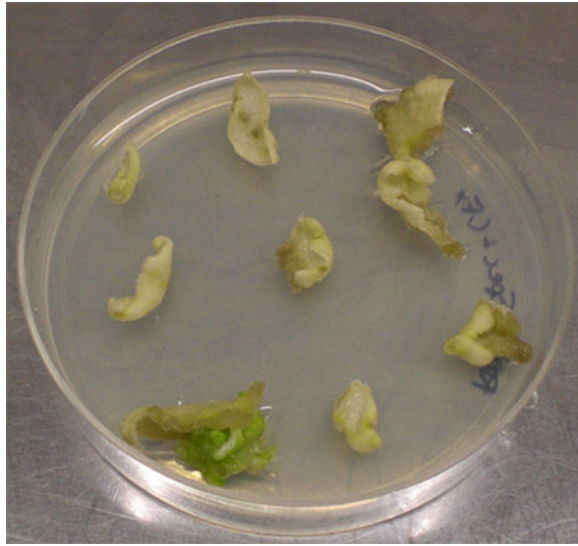


Fig. 1. Regeneration of a putative transformed shoot after 4–6 weeks of bombardment on RMOP selection medium (first round of regeneration).

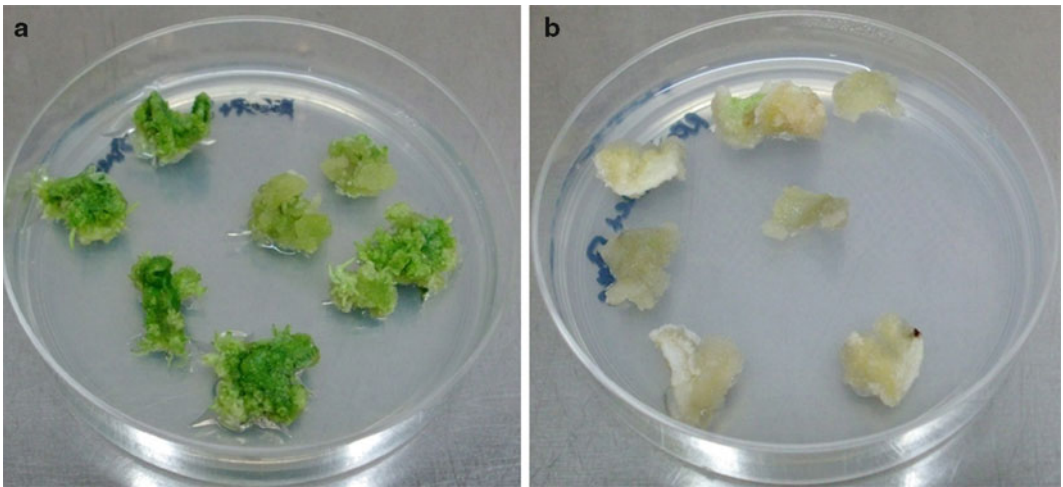


Fig. 2. Selection of primary regenerants on RMOP selection medium containing 500 mg/L of both spectinomycin and streptomycin. (a) positive explants, (b) negative explants.

3. Transfer the secondary green shoots on MS30B5 medium containing 500 mg/L of spectinomycin in Magenta box for rooting under controlled condition (16-h light $40 \mu\text{E}/\text{m}^2/\text{s}$ and 8-h dark at 24°C). Within 3–4 weeks, rooted plants with three to four leaves will be obtained (Fig. 3). At this stage, leaf DNA will be extracted and used to select transplastomic homoplasmic plants by PCR and Southern blot analyses.



Fig. 3. Rooted secondary shoots (positive to both spectinomycin and spectinomycin/streptomycin selections) on MS30B5 medium 500 mg/L of spectinomycin ready for leaf DNA extraction.

4. If the plants are homoplasmic, transfer to soil for seed production.
5. If the plants are not homoplasmic, cut into small pieces and transfer onto RMOP selection medium for the third round of regeneration.

3.4. Identification of Transplastomic Plants by PCR

1. Extract total leaf DNA from wild-type (control) plants and putative transformed plants (see Note 5).
2. Perform PCR reaction (25 μ L) for each sample as follow: 50 ng of total DNA from putative transformants and control plant, 2.5 μ L of 10 $\times$ PCR buffer, 0.75 μ L of 50 mM $MgCl_2$, 0.5 μ L (20 μ M) of each primer, 0.5 μ L of 10 mM dNTPs, 1.25 units of Taq DNA polymerase, and adjust the final volume with sterile water.
3. Set the PCR reaction as follows: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55–60°C for 1 min, elongation at 72°C for 1 min /kb; and final elongation at 72°C for 5 min.
4. Analyze the PCR products by agarose gel (0.8% w/v) electrophoresis/ethidium bromide staining. PCR products from putative transplastomic plants show a variable size depending on the size of the foreign gene inserted and from primers selected for the analysis. The wild-type plants show no amplification product.

**3.5. Identification
of Homoplasmic
Plants by Southern
Blot Analysis**

1. Extract total leaf DNA from wild-type (control) plants and transformed plants (see Note 5).
2. Digest 1.5–2.5 μg of total DNA from transformed and control plants with suitable restriction enzymes and incubate at suggested temperature overnight.
3. Run digested DNA and DNA molecular weight marker on 0.8% agarose gel, take a picture of your gel, and nick a corner to define the orientation of the gel.
4. Depurinate DNA in 0.25 N HCl for 10 min by shaking gently and rinse twice with distilled water.
5. Denature DNA in transfer buffer (0.4 N NaOH) for 10–15 min by shaking gently.
6. Turn the gel upside down. Prewet the nylon membrane (nick a corner to define the orientation of the membrane) in transfer solution, place it on top of the gel, and smooth out bubbles. Put on the top of the membrane six pieces of Whatman paper and blotting paper. Apply a weight of 500 g on top and allow the DNA to transfer overnight.
7. Remove the membrane with transferred DNA (use a pencil to mark the wells) and cross-link it by using GS GeneLinker UV Chamber (C3 program).
8. Prepare flanking probe by PCR amplification of the flanking sequences using digoxigenin-11 dUTP. Perform PCR reaction (50 μL) as follows: 1–50 ng of total DNA from wild-type plant, 5 μL of 10 $\times$ PCR buffer, 1.5 μL of 50 mM MgCl_2 , 1 μL (20 μM) of each primer, 5 μL of DIG dNTPs (final concentration 200 μM dATP, dCTP, and dGTP, 130–185 μM dTTP, and 15–70 μM DIG-dUTP, see Note 2), 2.5 units of Taq DNA polymerase, and adjust the final volume with sterile water. Prepare a tube for unlabeled control probe using a standard dNTP mixture.
9. Set the PCR reaction as follows: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 55–60°C for 1 min, elongation at 72°C for 1 min /kb; and final elongation at 72°C for 7 min.
10. Analyze the PCR products by agarose gel (0.8% w/v) electrophoresis/ethidium bromide staining. The labeled probe will migrate slower than unlabeled control probe due to the presence of digoxigenin (DIG).
11. Preheat at 37–42°C an appropriate volume (10 mL/100 cm² membrane) of DIG Easy Hyb and prehybridize membrane for at least 30 min by shaking gently (see Note 6 to calculate hybridization temperature).

12. Denature DIG-labeled probe (25 ng/mL DIG Easy Hyb) by boiling for 5 min and cool in ice. Add denatured DIG-labeled probe to preheated DIG Easy Hyb (3.5 mL/100 cm² membrane) and mix.
13. Pour off prehybridization solution, add probe/hybridization mixture to membrane, and incubate overnight by shaking gently.
14. Wash the membrane twice with 2× SSC, 0.1% SDS at room temperature for 5 min by shaking.
15. Wash the membrane twice with preheated 0.5× SSC, 0.1% SDS at 65–68°C for 15 min by shaking.
16. Rinse the membrane briefly in washing buffer.
17. Incubate the membrane for 30 min in 100 mL blocking solution at room temperature by shaking.
18. Incubate the membrane for 30 min in 20 mL antibody solution at room temperature by shaking.
19. Wash the membrane twice in 100 mL washing buffer for 15 min at room temperature by shaking.
20. Equilibrate the membrane in 20 mL detection buffer for 15 min at room temperature.
21. Place membrane with DNA side facing up on a plastic wrap and apply 1 mL CSPD ready to use. Immediately cover the membrane with plastic wrap, spread the substrate over the membrane, and smooth out bubbles. Incubate at room temperature for 5 min.
22. Squeeze out excess liquid and incubate the damp membrane for 10 min at 37°C to enhance the luminescent reaction.
23. Expose to X-ray film for 15–30 min.

3.6. Extraction of Total Soluble Proteins

1. Grind 50–100 mg leaf and add 200–400 µL of freshly prepared PBS HS on ice.
2. Homogenize for 5 min the tissue by using polypropylene pestle and keeping the sample in ice to prevent protein degradation. Vortex briefly.
3. Centrifuge at 11,200×*g* for 10 min at 4°C.
4. Aliquot the supernatant (soluble proteins) and proceed to protein quantitation by Bradford assay.

3.7. Western Blot Analysis to Evaluate the Expression of Recombinant Proteins

1. Prepare a 12% separating gel (1.5-mm thick) using a Mini PROTEAN 3 system (Bio-Rad) by mixing 3.375 mL water, 1.875 mL separating buffer (4×), 2.25 mL acrylamide/bis solution, 37.5 µL 10% ammonium persulfate solution, and 7.5 µL TEMED. Pour the solution smoothly, leaving space for the stacking gel, and overlay with isopropanol. Gel polymerization should take 45–60 min.

2. Pour off the isopropanol, rinse the gel surface with water, and dry the area between glass plates with a piece of Whatman paper.
3. Prepare the stacking gel by mixing 3.075 mL water, 1.25 mL stacking buffer (4×), 625 μ L acrylamide/bis solution, 50 μ L 10% ammonium persulfate solution, and 10 μ L TEMED. Pour the solution until the top of the short plate is reached, and then insert the desired comb between the spacers. Gel polymerization should take 30–40 min.
4. Prepare 1 L of running buffer 1× (1:5 diluted from 5× solution).
5. Remove the comb and wash the wells with water and running buffer using a syringe.
6. Prepare the samples (plant extracts and standard protein) diluting them in loading sample buffer and boil for 5–10 min.
7. Put immediately the samples on ice and load them and protein marker into wells of 12% SDS-polyacrylamide gel.
8. Run the gel with initial current at 80 V until proteins migrate into separating gel, then increase the current at 150 V. Stop the run when the dye reaches the bottom of the separating gel.
9. Transfer the separated proteins to nitrocellulose membrane in blotting buffer 1× for 1 h at 100 V.
10. Remove the membrane and rinse with 1× PBS.
11. Incubate the membrane in blocking buffer for 2 h at room temperature or overnight at 4°C by shaking.
12. Pour off the blocking buffer and wash with T-PBSW for 10 min by shaking.
13. Add primary antibody diluted in blocking buffer (see Note 7) and incubate at room temperature for 2 h by shaking.
14. Wash the membrane with T-PBSW for 10 min by shaking (repeat three times).
15. Add secondary antibody (anti-rabbit) diluted (1:60,000) in 1× PBS containing 5% nonfat dry milk and incubate at room temperature for 1 h by shaking.
16. Wash the membrane with T-PBSW for 10 min by shaking (repeat three times).
17. Add ECL substrate, spread it on the membrane, and incubate for 5 min at room temperature.
18. Squeeze out excess liquid and expose the membrane to ECL film for about 5 min (Fig. 4).

**3.8. Enzyme-Linked
Immunosorbent Assay
to Quantify the
Expression Level of
Recombinant Proteins**

The procedure described is referred to a sandwich (or capture) ELISA that measures the amount of antigen between two layers of antibodies. This procedure is particularly indicated when the concentration of antigen is contained in high concentrations of contaminating proteins (e.g., crude plant extracts). The crude plant

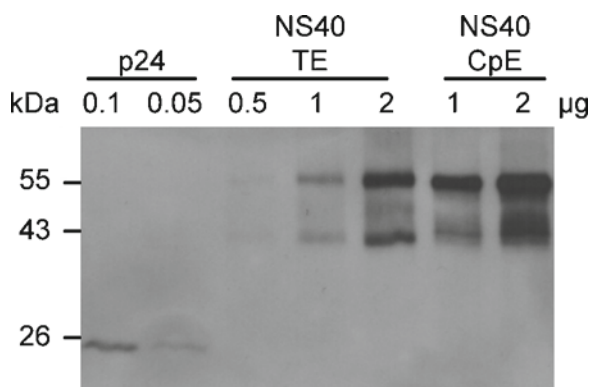


Fig. 4. Western blot analysis of total protein extracts (TE) and chloroplast protein extracts (CpE) of transplastomic NS40 plant overexpressing the HIV-1 Pr55^{gag} polyprotein using a rabbit polyclonal antibody against p24. p24 = recombinant p24 protein produced in *E. coli* (0.1 and 0.05 μg); NS40-TE = total soluble proteins from *N. tabacum* transformed with pNS40 vector (0.5, 1, and 2 μg); NS40-CpE = chloroplast proteins from *N. tabacum* transformed with pNS40 vector (1 and 2 μg).

proteins used for this procedure were extracted using 1× PBS HS without DTT (native condition).

1. Coat the plate with 50 μL/well of monoclonal antibody anti-Pr55/p24 (ARP 3243.4) diluted in 1× PBS (2.5 mg/mL) and incubate the plate overnight at 4°C.
2. Pour off the solution and wash the plate twice with T-PBSE.
3. Block the plate with 150 μL/well of T-PBSEM5 for 1.5–2 h at 37°C.
4. Pour off the solution and wash the plate twice with T-PBSE.
5. Add 50 μL/well of T-PBSEM1, then sample crude extract or antigen standard, and incubate for 1 h at 37°C.
6. Pour off the samples and wash the plate three times with T-PBSE.
7. Add 50 μL/well of polyclonal anti-p24 antibody (ARP432) diluted in T-PBSEM1 (1:5,000) and incubate for 1 h at 37°C.
8. Pour off the solution and wash the plate three times with T-PBSE.
9. Add 50 μL/well of polyclonal anti-rabbit antibody conjugated to horse radish peroxidase diluted in T-PBSEM1 (1:5,000) and incubate for 1 h at 37°C.
10. Pour off the solution and wash the plate four times with T-PBSE.
11. Develop with TMB substrate (50 μL/well) for 5–10 min.
12. Stop the reaction by adding (50 μL/well) of 2 N sulfuric acid.
13. Read the plate on a microtiter plate reader using 450-nm filter.

3.9. Extraction of Chloroplast Proteins by Differential Centrifugation

This protocol describes a rapid procedure to isolate chloroplasts (based on differential centrifugations) and extract total proteins from enriched chloroplast fraction. It generally ensures a higher yield than methods based on the isolation of purified chloroplasts on percoll or sucrose gradients, although more contaminations are present. All operations are carried out at 4°C.

1. Keep plants in the dark overnight before harvesting (see Note 8).
2. Homogenize 4–20 g of leaves with 16–80 mL of homogenization buffer two times for 5 s in a Waring blender at 4°C (see Note 9).
3. Filter the homogenate rapidly through two layers of Miracloth.
4. Centrifuge at $500 \times g$ for 5 min at 4°C.
5. Recover the supernatant and centrifuge it twice at $2,600 \times g$ for 15 min and 10 min (the second time) at 4°C.
6. Resuspend the pellets, carefully, with 10 mL of homogenization buffer.
7. Centrifuge the chloroplast solution at $2,600 \times g$ for 15 min at 4°C.
8. Pour off the supernatant and resuspend the pellet with 1.2–6 mL of lysis buffer on ice.
9. Leave on ice for 10 min and then vortex vigorously.
10. Aliquot the crude chloroplast proteins and proceed to protein quantitation by Bradford assay.

4. Notes

1. Spermidine is very hygroscopic and air sensitive. Prepare stock solution and aliquot into small volumes to avoid freeze and thaw. After use, discard the remaining volume.
2. The production of the flanking probe by PCR amplification using DIG-dUTP can require an adjustment of the DIG-dUTP concentration, and generally, 70 μM as final concentration of DIG-dUTP works well for labeling probes up to 1 kb long; reduce the final concentration to 35 μM for probes 1–3 kb long. The labeling of probes >3 kb can require the use of a Long Taq DNA polymerase and a gradual reduction of the final concentration of DIG-dUTP up to 7 μM (depending on the specific-target sequences).
3. SDS and PMSF are harmful by inhalation or in contact with skin and can cause irritation to the eyes, skin, and respiratory system.
4. Place no more than nine pieces per Petri dish because leaf segments expand during in vitro culture.

5. Total DNA can be isolated by both kit and standard procedure (e.g., CTAB).
6. The appropriate hybridization temperature is calculated according to manufacturer's instructions and should be 20–25°C below the calculated T_m . T_m is calculated according the following equation:

$$T_m = 49.82 + 0.41(\%G + C) - (600 / l)$$

[l = length of hybrid in base pairs].

7. The appropriate dilution of the primary antibody are the following: 1:10,000 for anti-p24 antibody (ARP432, NIBSC) and 1:15,000 for anti-p17 antibody (ARP431, NIBSC).
8. Starch granules present in chloroplasts interfere with the isolation of intact organelle. In fact, chloroplasts containing large starch grains are generally broken during centrifugation. Therefore, prior to proceeding with the experiment, the plants should be kept in the dark to reduce the amount of starch.
9. The homogenization of plant material with the Waring blender must be as short as possible to reduce the portion of broken chloroplasts.

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Use of a Callus-Specific Selection System to Develop Transgenic Rice Seed Accumulating a High Level of Recombinant Protein

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Abstract

A mutated rice acetolactate synthase (*mALS*) gene expressed under the control of the rice callus-specific promoter (CSP) (CSP:*mALS*) becomes a useful selectable marker for producing transgenic rice seed with higher accumulation of recombinant protein. When amounts of transgene products in mature seeds are compared between transgenic rice lines containing the CSP:*mALS* selection gene cassette and those with the hygromycin phosphotransferase (HPT) gene under the control of the CaMV 35S promoter (35S:*HPT*), the former transgenic rice seeds usually resulted in 1.2- to 2-fold higher accumulation of transgene products than in the latter. It is considered that specific expression of a selection marker gene at the selection stage may allow enhanced transgene products in seeds.

This chapter represents a highly efficient *Agrobacterium*-mediated rice transformation system using the CSP:*mALS* gene cassette in place of the conventional constitutive selection using bacterial antibiotics. This selection stage specific expression using a rice-derived selection marker *mALS* will be especially beneficial for developing transgenic rice seeds accumulating bioactive proteins or peptides contributing to human health promotion; pharmaceutical products such as vaccine, antibodies, and biopharmaceuticals; and industrial enzymes.

Key words: *Agrobacterium*, Mutated acetolactate synthase, Callus-specific promoter, *Oryza sativa* L., Selectable marker, Transgenic rice

1. Introduction

Rice seed is an attractive production platform (bioreactor) for recombinant proteins. When expressed under the control of a seed-specific promoter, the recombinant protein can be highly produced at the desired site within the seed, as determined by the property of the particular seed-specific promoter. The recombinant proteins accumulated in seed are stable for several years even if stored at

room temperature. Furthermore, the rice seed production system also has advantages in economy, scalability, and safety over the conventional expression systems using bacteria, insect, and mammalian cells (1, 2).

When transgenic rice plants are generated by using a conventional method, bacterial genes encoding hygromycin phosphotransferase (*HPT*), phosphinothricin acetyltransferase (*BAR*), or neomycin phosphotransferase (*NPT II*) under the control of the constitutive CaMV 35S, maize ubiquitin-1, or rice actin promoters have been used as selectable markers to distinguish between transgenic and nontransgenic rice cells. However, such selectable antibiotic resistance genes derived from *E. coli*, or other bacteria, have been a focus of the debate over the safety of genetically modified crops (GM crops).

Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids leucine, isoleucine, and valine. The sulfonylurea, imidazolinone, and pyrimidinyl carboxy herbicides inhibit ALS activity by acting as competitive inhibitors (3, 4), and a *mutated ALS* (*mALS*) gene has been isolated from mutant plants resistant to these herbicides. The *mALS* enzyme has much lower affinity for these herbicides in transgenic plant cells, but has the same activity as ALS, and has been used to generate a number of herbicide-resistant transgenic plant species (5–8). Transgenic rice plants have been produced using *Arabidopsis mALS* under the control of the CaMV 35S promoter as a selectable marker (7). Rice *mALS* alleles were also isolated from a pyrimidinyl carboxy herbicide (pyriminobac, pyriithiobac-sodium, and bispyribac-sodium)-resistant rice mutant (4, 9). Transgenic rice produced using the *mALS* gene as a selectable marker may be more acceptable to consumers, since this selectable marker gene is derived from the rice genome. Furthermore, the new selection system introduced in this chapter permits the production of transgenic rice by using rice *mALS* under the control of the rice callus-specific promoter (CSP:*mALS*). Because *mALS* transcripts driven by CSP are not detected in tissues other than callus, the food safety of transgenic rice seeds may be significantly improved from the perspective of a consumer.

Here, it is important to note that accumulation levels of transgene products are increased in transgenic rice seeds by expressing a selection marker gene only at the callus selection stages, when compared with transgenic plants selected by the conventional constitutive selection marker genes (10, 11). Therefore, development of transgenic rice plant by using CSP:*mALS* has two distinct advantages. One is that scientists and molecular breeders can improve the perception of safety of the transgenic rice to consumers because *mALS* is not expressed in any edible parts such as the vegetative tissues and seed. The other advantage is enhanced accumulation levels of transgene products.

In this chapter, the method for generating transgenic rice using CSP:*mALS* as a selection marker will be introduced. This method usually has a transformation efficiency of at least 30% and will be useful for developing commercial transgenic rice plants.

2. Materials

2.1. Preparation of *Agrobacterium* Competent Cells

1. *Agrobacterium* (EHA105, etc.).
2. YEB liquid medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L tryptone peptone, 5 g/L sucrose, 2 mM MgSO₄).
3. YEB solid medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L tryptone peptone, 5 g/L sucrose, 2 mM MgSO₄, 1.5% agar).
4. 10% Glycerol.
5. Sterilized water.
6. Antibiotics (20 mg/mL rifampicin, etc.).
7. Liquid N₂.

2.2. Electrotransformation of *Agrobacterium*

1. *Agrobacterium* (EHA105, etc.) competent cell.
2. YEB liquid medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L tryptone peptone, 5 g/L sucrose, 2 mM MgSO₄).
3. YEB solid medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L tryptone peptone, 5 g/L sucrose, 2 mM MgSO₄, 1.5% agar).
4. Antibiotics (50 mg/mL kanamycin, 100 mg/mL spectinomycin, etc.).

2.3. Tissue Culture

1. N6D medium [4.0 g/L CHU (12) basal salt mixture, N6-vitamins (12), 2 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 300 mg/L casamino acids, 2.88 g/L proline, 4.0 g/L gelrite] (pH 5.8).
2. AAM medium (1 mL/L AA-1, 1 mL/L AA-2, 1 mL/L AA-3, 1 mL/L AA-4, 1 mL/L AA-5, 5 mL/L AA-6, 10 mL/L AA-7,¹ 0.5 g/L casamino acid, 68.5 g/L sucrose, 36 g/L glucose, 0.9 g/L L-glutamine, 0.3 g/L L-aspartic acid, 3 g/L KCl) (pH 5.2).

¹100 mL AA-1 (1 g MnSO₄·4H₂O, 0.3 g H₃BO₄, 0.2 g ZnSO₄·7H₂O, 25 mg Na₂MoO₄·2H₂O, 2.5 mg CuSO₄·5H₂O, 2.5 mg CoCl₂·6H₂O, 75 mg KI), 100 mL AA-2 (15 g CaCl₂·2H₂O), 100 mL AA-3 (25 g MgSO₄·7H₂O), 100 mL AA-4 (4 g Fe-EDTA), 100 mL AA-5 (15 g NaH₂PO₄·2H₂O), 100 mL AA-6 (20 mg nicotinic acid, 200 mg thiamine hydrochloride, 20 mg pyridoxine monohydrochloride, 2 g *myo*-inositol), 100 mL AA-7 (1.77 g L-arginine, 95 mg glycine).

3. 2N6-AS medium [4.0 g/L CHU basal salt mixture, N6-vitamins, 2 mg/L 2,4-D, 30 g/L sucrose, 10 g/L glucose, 100 mg/L *myo*-inositol, 300 mg/L casamino acids, 2 mg/L acetosyringone, 4.0 g/L gelrite] (pH 5.2).
4. MS regeneration medium [4.6 g/L MS (13) basal salt mixture, B5-vitamins (13), 0.2 mg/L NAA, 2 mg/L kinetin, 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 4 g/L gelrite] (pH 5.8).
5. Modified-DKN medium (14) [50 mL/L DKN-macro, 1 mL/L DKN-micro, 1 mL/L R2-iron, modified B5-vitamins,² 2 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 300 mg/L casamino acids, 1.15 g/L proline, 670 mg/L L-aspartic acid, 730 mg/L L-glutamine, 4 g/L gelrite] (pH 5.8).
6. DKN-AS medium [50 mL/L DKN-macro, 1 mL/L DKN-micro, 1 mL/L R2-iron, modified B5-vitamins, 2 mg/L 2,4-D, 30 g/L sucrose, 10 g/L glucose, 100 mg/L *myo*-inositol, 300 mg/L casamino acids, 2 mg/L glycine, 670 mg/L L-aspartic acid, 730 mg/L L-glutamine, 2 mg/L acetosyringone, 4 g/L gelrite] (pH 5.2).
7. Modified-DKN regeneration medium [50 mL/L DKN-macro, 1 mL/L DKN-micro, 1 mL/L R2-iron, modified B5-vitamins, 0.2 mg/L NAA, 2 mg/L kinetin, 30 g/L sucrose, 30 g/L sorbitol, 100 mg/L *myo*-inositol, 300 mg/L casamino acids, 1.15 g/L proline, 2 mg/L glycine, 670 mg/L L-aspartic acid, 730 mg/L L-glutamine, 4 g/L gelrite] (pH 5.8).
8. MS-hormone-free medium [4.6 g/L MS basal salt mixture, B5-vitamins, 30 g/L sucrose, 2.5 g/L gelrite] (pH 5.8).
9. Sterilized water.
10. Sodium hypochlorite solution.
11. Pyriminobac (Kumiai Chemical Co., Tokyo, Japan) (see Note 1).
12. Carbenicillin or meropenem.
13. AB medium [3 g/L K_2HPO_4 , 1.3 g/L $NaH_2PO_4 \cdot 2H_2O$, 1 g/L NH_4Cl , 150 mg/L KCl, 10 mg/L $CaCl_2 \cdot 2H_2O$, 2.5 μ g/L $FeSO_4 \cdot 7H_2O$, 500 mg/L $MgSO_4 \cdot 7H_2O$, 5 g/L glucose, antibiotics (e.g., 50 mg/L kanamycin, 100 mg/L spectinomycin), 1.5% agar] (see Note 2).

²1 L DKN-macro (20 $\times$ stock) (5.46 g NaH_2PO_4 , 16 g KNO_3 , 1.34 g $(NH_4)_2SO_4$, 5 g $MgSO_4$, 3 g $CaCl_2 \cdot 2H_2O$), 100 mL DKN-micro (1,000 $\times$ stock) (0.16 g $MnSO_4 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.3 g H_3BO_3 , 12.5 mg $CuSO_4 \cdot 5H_2O$, 12.5 mg $Na_2MoO_4 \cdot 2H_2O$), 100 mL modified B5-vitamins (1,000 $\times$ stock) (100 mg nicotinic acid, 1 g thiamine hydrochloride, 100 mg pyridoxine monohydrochloride, 10 g *myo*-inositol, 200 mg glycine), 100 mL R2-iron (1,000 $\times$ stock) (750 mg Na_2 -EDTA, 550 mg $FeSO_4 \cdot 7H_2O$).

2.4. Protein Extraction from Mature Seed

1. Extraction buffer (50 mM Tris-HCl pH 6.8, 8 M urea, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue).

3. Method**3.1. Preparation of *Agrobacterium* Competent Cells for Electrotransformation**

1. Pre-culture *Agrobacterium* onto YEB solid medium plus 20 mg/L rifampicin at 28°C for 2 days.
2. Inoculate 3 mL of YEB medium plus 20 mg/L rifampicin with a single colony of *Agrobacterium* and incubate overnight at 28°C with vigorous shaking.
3. Add 3 mL of overnight culture to 100 mL of YEB medium plus 20 mg/L rifampicin in a 500-mL flask and incubate for 12–14 h at 28°C with vigorous shaking.
4. Pellet the *Agrobacterium* by centrifugation at 3,000×*g* for 10 min at 4°C.
5. Wash the *Agrobacterium* pellet with cold sterilized water on ice. Repeat five times steps 4 and 5.
6. Wash the *Agrobacterium* pellet with cold 10% glycerol on ice and pellet the *Agrobacterium* as step 4.
7. Resuspend the *Agrobacterium* pellet with 4 mL of 10% glycerol.
8. Dispense the *Agrobacterium* competent cell to a new tube (40 µL/tube) and immediately freeze by using liquid N₂. Afterwards, store in a –80°C freezer.

3.2. Electrotransformation of *Agrobacterium*

1. Mix the 1 µL of binary vector solution (1–100 ng) to 40 µL of *Agrobacterium* competent cells and transfer the mixture into a cold cuvette. Place the cuvette in an *E. coli* Pulser apparatus as described in the manufacturer's protocol.
2. Set the *E. coli* Pulser apparatus to 2.5 kV when 0.2-cm cuvettes are used.
3. Push the pulse switch once, then immediately add 1 mL of YEB medium to the cuvette, and gently suspend the *Agrobacterium*. Transfer the suspension to a new tube and incubate at 28°C for more than 1 h.
4. Spread the suspension onto the YEB solid medium with antibody and incubate at 28°C for 2 days. Obtain single colonies.

3.3. Callus Induction from Seed Scutellum

Optimum conditions for tissue culture differ among rice cultivars. In Japonica rice, tissue culture conditions fall into two main categories. One is appropriate for transformation of most Japonica rice, and the other is for Koshihikari and its variants.

Koshihikari is the major cultivated variety in Japan due to the good taste of its grain. This makes Koshihikari a desirable target for molecular breeding using transgenic techniques. However, Koshihikari has not been used as a host for transgenes because it does not readily produce callus tissue, making it extremely difficult to regenerate transgenic plants. This difficulty has been traced to the low expression of ferredoxin-nitrite reductase (*NiR*) in callus (14). Daigen and co-workers showed that Koshihikari calli grew well in DKN medium in which amino acids are the main nitrogen source (15). These findings indicate that nitrogen metabolism is an important factor for regeneration from Koshihikari calli. Ozawa and Kawahigashi have recently developed a novel *Agrobacterium*-mediated transformation method for Koshihikari using N6D and DKN media (16).

We mainly present here a transformation method for Japonica rice by using CSP:*mALS* selectable marker. As regards Indica rice, although successful transformation by using CSP:*mALS* has not yet been reported, it is probably possible to produce transgenics using the same conditions as for Japonica varieties. We consider that previous research papers about the conventional method for Indica rice transformation (17, 18) would provide valuable information. Moreover, the modified method for Japonica rice transformation as described by Ozawa (19) may be useful for improvement of the transformation efficiency (for further information, please refer to Chapter 5 by Ozawa).

3.3.1. Most Varieties of Japonica Rice

1. Prepare about 100 seeds and remove their glumes (see Note 3). Collect seeds in a 50-mL tube.
2. Add 20 mL of distilled water and the same volume of sodium hypochlorite solution in the tube. Gently shake for 30 min at room temperature.
3. Wash seeds five times with sterilized water by inverting the tube.
4. Put about 20 seeds onto a plate containing N6D medium (Fig. 1a) and culture for 4 weeks at 32°C (16 h light/8 h dark) (Fig. 1c).

3.3.2. Some Types of Japonica Rice (E.g., Koshihikari or Related Variety)

1. Same protocol as above steps 1–3.
2. Put 20 seeds onto a plate containing N6D medium and culture for 1 week at 32°C (16 h light/8 h dark).
3. Remove small callus generated from the seed scutellum (Fig. 1b) and transfer the selected calli onto a plate containing DKN medium.
4. Incubate for 3 weeks at 32°C (16 h light/8 h dark) (Fig. 1c).

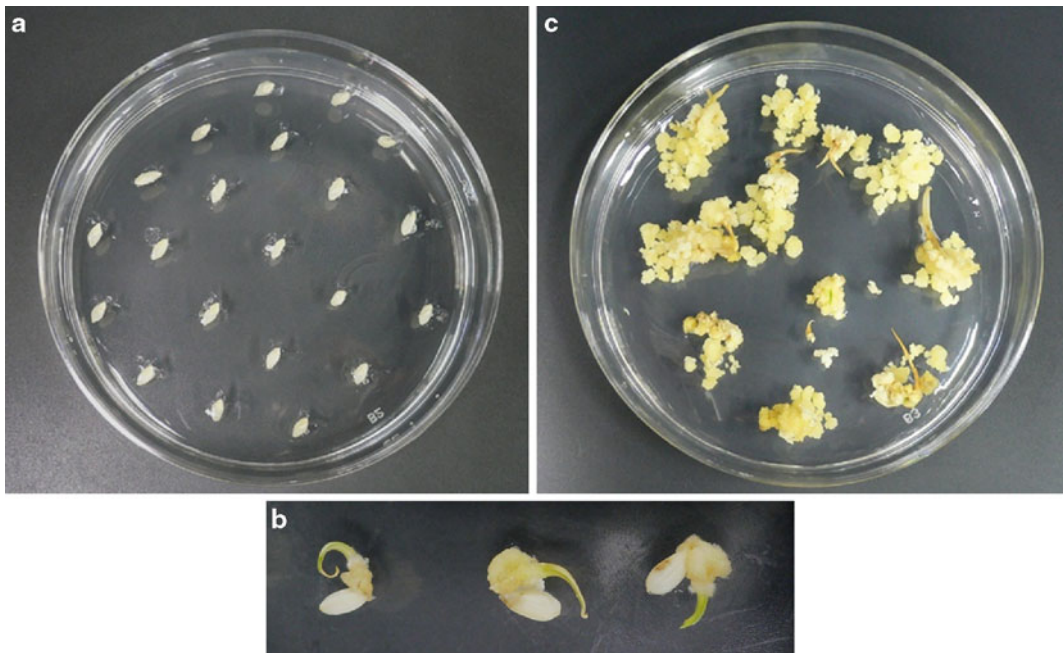


Fig. 1. Sowing of seed on N6D medium for callus induction and the state of seed 7 days after sowing on N6D medium in Koshihikari. (a) Embryo directly in contact with N6D medium. (b) After 7 days, 1–3-mm calli generated from scutellum (middle and right seeds). If no or very small callus (left seed) is generated 7 days after sowing, the seed should be removed. (c) Induced callus (3–4 weeks after seedling on callus induction medium).

3.4. *Agrobacterium* Inoculation to Callus and Co-culture

3.4.1. Most Varieties of Japonica Rice

1. Pick up an *Agrobacterium* single colony from YEB medium and swab it onto AB medium with antibiotics. Incubate at 28°C for 3 days and obtain fresh *Agrobacterium* cells.
2. Suspend a half of small spatula of *Agrobacterium* colony into 30 mL of AAM medium with 2 mg/L acetosyringone in a 50-mL tube (Fig. 2a). (AAM medium should not become cloudy after *Agrobacterium* suspension).
3. Contact callus with the *Agrobacterium* suspension (approximately 100 calli per 10 mL *Agrobacterium* suspension) and incubate for 2 min (Fig. 2b).
4. Transfer *Agrobacterium*-contacted calli onto filter paper in a Petri dish and remove the surplus *Agrobacterium* suspension (Fig. 2c).
5. Prepare the 2N6-AS medium and put a small filter paper on the medium (Fig. 2d).
6. Transfer inoculated calli onto the filter paper and co-culture at 23–25°C for 3 days in darkness (Fig. 2e).

3.4.2. Some Types of Japonica Rice (E.g., Koshihikari or Related Variety)

1. Same protocol as above steps 1–4.
2. Prepare the DKN-AS medium and put a filter paper on the medium.

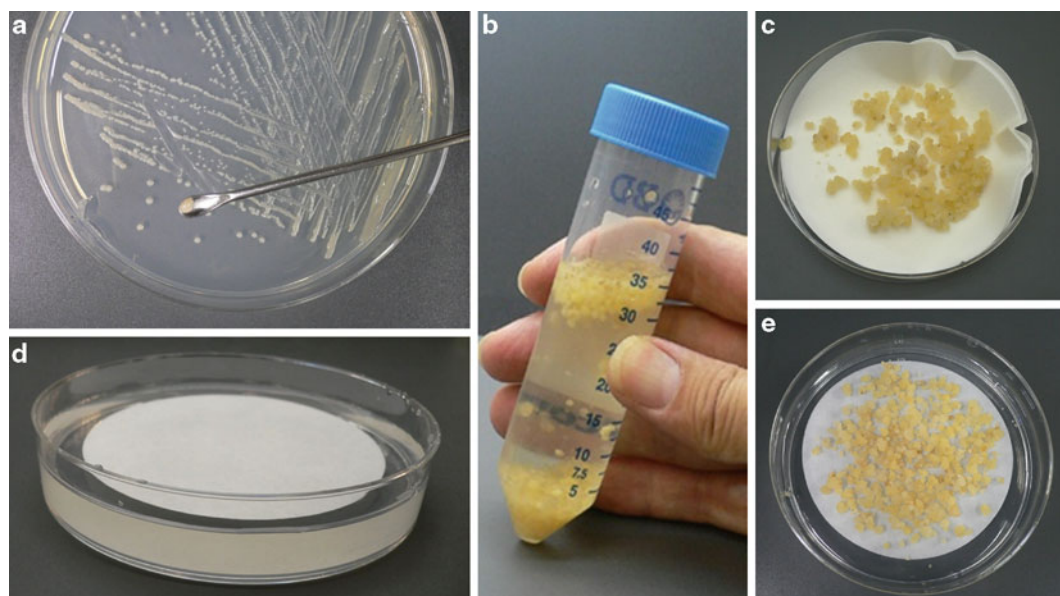


Fig. 2. *Agrobacterium* inoculation. (a) *Agrobacterium* growing on plate. (b) Contacting rice calli with *Agrobacterium*. (c) *Agrobacterium* suspension removed by blotting on filter paper. (d) Use of filter paper one size smaller than Petri dish. (e) Calli placed onto small filter paper.

3. Transfer inoculated calli onto filter paper and co-culture at 23–25°C for 3 days in darkness.

3.5. Callus Selection

3.5.1. Most Varieties of Japonica Rice

1. After co-culture, wash the infected calli five times with sterilized water and three times with sterilized water containing 400 mg/L carbenicillin or 100 mg/L meropenem (Fig. 3a) (see Note 4).
2. Transfer the calli onto filter paper in Petri dish and remove the surplus of washing solution.
3. Put about 20 calli onto N6D medium with 400 mg/L carbenicillin and suitable concentration of pyriminobac and culture at 32°C for 5–7 weeks (16 h light/8 h dark) (Fig. 3b, c). For suitable concentration of selection reagents (pyriminobac), please refer to Subheading 3.7.

3.5.2. Some Types of Japonica Rice (E.g., Koshihikari or Related Variety)

1. Same protocol as above steps 1 and 2.
2. Culture at 32°C for 5–7 weeks on DKN selection medium with 400 mg/L carbenicillin and suitable concentration of pyriminobac (16 h light/8 h dark).

3.6. Regeneration

3.6.1. Most Varieties of Japonica Rice

1. Transfer the pyriminobac-resistant calli to MS regeneration medium with 400 mg/L carbenicillin only and culture at 28°C to regenerate shoots and roots for 3–5 weeks (Fig. 4a) (see Notes 5 and 6).

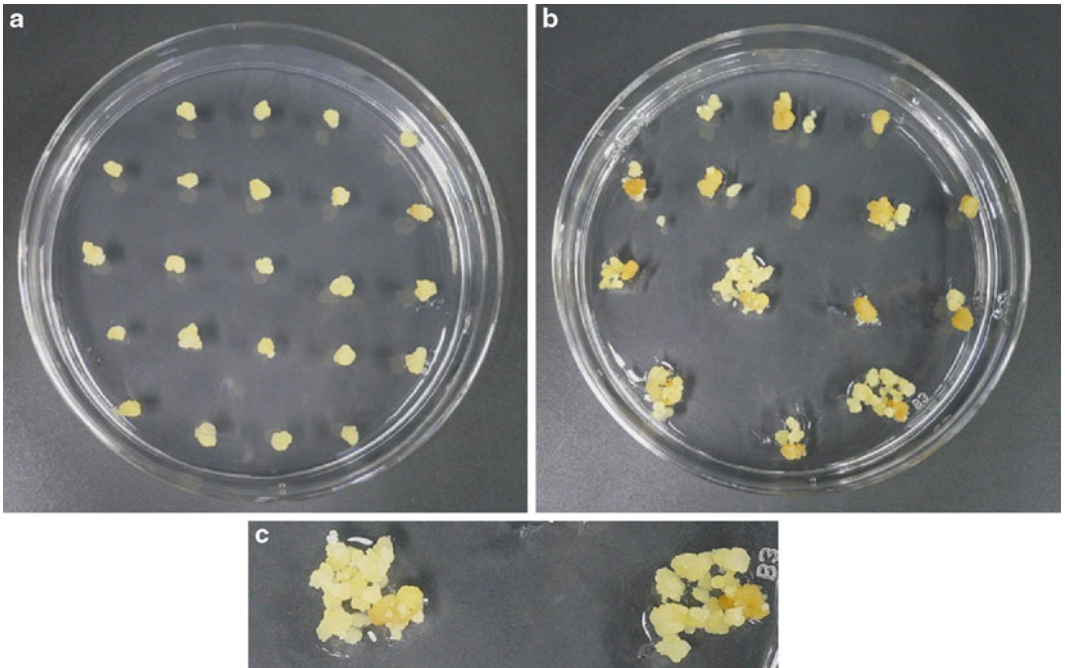


Fig. 3. Callus selection. (a) Callus selection medium. (b) Growth of pyriminobac-resistant calli. (c) Enlargement image of (b). Only vivid yellow calli should be used for regeneration.

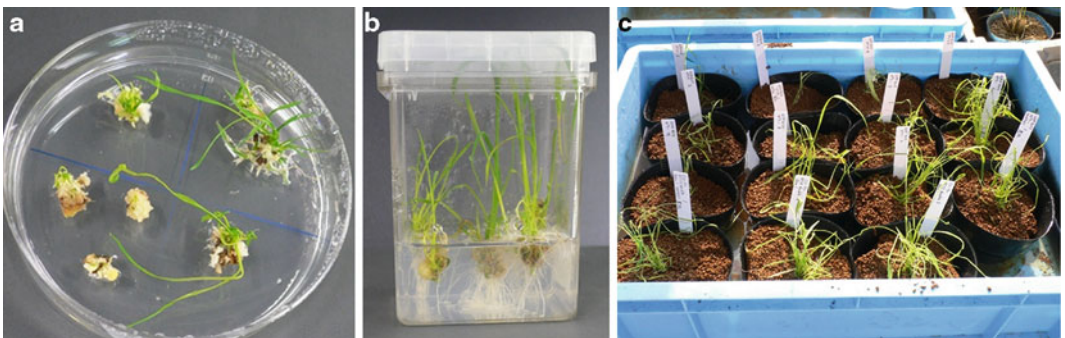


Fig. 4. Regeneration. (a) Calli approximately 4 weeks after growth on regeneration medium. (b) Regenerated plants with roots after transfer to MS-hormone-free medium. (c) Plantlets in closed green house.

2. Put regenerated small plant onto MS-hormone-free medium in a plant box and culture for elongation of shoot and root (Fig. 4b).
3. Transplant the regenerated plant to soil in a pot (Fig. 4c) (see Note 7).

3.6.2. *Some Types of Japonica Rice (E.g., Koshihikari or Related Variety)*

1. Transfer the pyriminobac-resistant calli to modified-DKN regeneration medium with 400 mg/L carbenicillin only and culture at 28°C for 1 week.
2. Transfer calli to MS regeneration medium with 400 mg/L carbenicillin only and culture at 28°C to regenerate shoots and roots (see Note 8).
3. Put regenerated small plant onto MS-hormone-free medium in plant box and culture for elongation of shoot and root.
4. Transplant the regenerated plant to soil in a pot.

3.7. **Examination of Pyriminobac Concentration for Suitable Callus Selection by Using GFP Under the Control 35S Promoter**

It is important to note that suitable concentrations of pyriminobac vary depending on the used rice cultivar. For example, concentrations of 2.5–3.0 μM are required for selection of transgenic Kitaake and Nipponbare calli. On the other hand, 1.0 μM pyriminobac has enough effect on nontransformed Koshihikari and Koshihikari variety calli. Although bispyribac-sodium and pyriithiobac-sodium can be used in place of pyriminobac, the effective concentrations may also vary among these herbicides. Therefore, examination of selection pressure is necessary for suitable transformation efficiency.

1. Transform a binary vector harboring CaMV 35S promoter GFP gene cassette (Fig. 5a) into the *Agrobacterium* cell.
2. Perform *Agrobacterium* infection, co-culture, and washing, as described in Subheadings 3.4 and 3.5.
3. Put *Agrobacterium*-infected callus on selection medium with various concentration of pyriminobac (e.g., 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 μM).

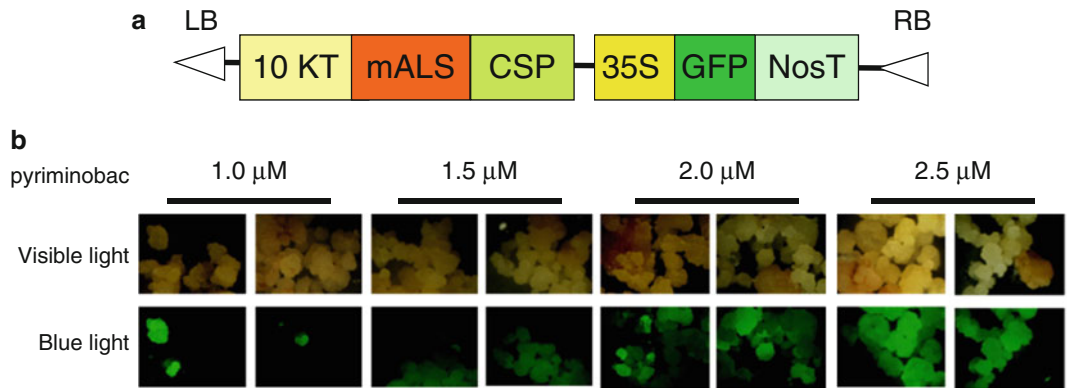


Fig. 5. Pyriminobac concentrations for transformed calli selection using GFP as a reporter gene. (a) Binary vector construct of GFP under the control of constitutive promoter. *CSP* callus-specific promoter, *mALS* mutated ALS coding, *10 KT* 10 kD prolamin gene terminator, *35S* CaMV 35S promoter, *GFP* green fluorescent protein, *NOS* nopaline synthase terminator. (b) Callus proliferation on callus selection medium with pyriminobac at 1.0 μM , 1.5 μM , 2.0 μM , and 2.5 μM . Upper panels are visible light, and lower panels are blue light (GFP). In this case, 2.0–2.5 μM of pyriminobac is suitable for callus selection.

4. Observe the growth calli under the blue light condition and determine the suitable concentration of pyriminobac (Fig. 5b).

3.8. Protein Extraction from Mature Seed

1. Grind mature seeds individually into fine powder.
2. Add 500 μL of total protein extraction buffer [50 mM Tris-HCl pH 6.8, 8 M urea, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol] to seed powder and vigorously vortex for 1 h at room temperature.
3. Centrifuge the mixture at 14,000 rpm for 20 min at room temperature and collect the supernatant in a new tube.
4. Subject sample (usually 2–3 μL) to electrophoresis on SDS-PAGE and immunoblot analysis.

4. Notes

1. Although bispyribac-sodium and pyriithiobac-sodium can be used in place of pyriminobac, the effective concentrations may also vary among these herbicides. Thus, suitable concentrations of these selection reagents should be also determined as a primary experiment (please refer to Subheading 3.7).
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ should be added after autoclaving. Thus, prepare the 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution sterilized by 2 μm filtration; after autoclaving, add 2 mL of stock solution to unfinished AB medium.
3. Avoid wounding the seed surface when hull is removed because wounding of seed surface decreases the callus generation rate.
4. If water became quite turbid after first washing, you will have to do the *Agrobacterium* inoculation again. The water after first washing is usually almost transparent.
5. CSP:*mALS* gene cassette expresses in callus only. Shoot and root cannot survive on the regeneration medium containing pyriminobac.
6. Only the growing calli around the initial callus (callus put onto selection medium at callus selection start time) should be put onto the regeneration medium. Do not put the initial callus onto the regeneration medium.
7. If the regenerated plant is not vigorous, habituation should be performed for 3 days in a clean room before planting.
8. Regeneration from Koshihikari callus is difficult as compared with the other Japonica rice cultivars. Browning may occur during regeneration in Koshihikari callus. Browning callus has to be removed from medium since this has an inhibitory effect on

yellow (normal) callus growth. Only yellow callus should be kept on the regeneration medium until shoot and root regeneration.

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Part VIII

Field Tests

Chapter 37

How to Grow Transgenic *Arabidopsis* in the Field

Hanna Johansson Jänkänpää and Stefan Jansson

Abstract

Arabidopsis is naturally adapted to habitats in which both biotic variables (e.g., light, wind, and humidity) and abiotic variables (e.g., competition, herbivory, and pathogen densities) strongly fluctuate. Hence, conditions in controlled growth chambers (in which *Arabidopsis* is typically grown for scientific experiments) differ substantially from those in natural environments. In order to mimic more closely natural conditions, we grow *Arabidopsis* outdoors under “semi-natural” field conditions. Performing experiments on transgenic *Arabidopsis* grown in the field that are sufficiently reliable for publication is challenging. In this chapter, we present some of our experiences based on 10 years of field experimentation, which may be of use to researchers seeking to perform field experiments using transgenic *Arabidopsis*.

Key words: *Arabidopsis thaliana*, Transgenic lines, Field experiments, Fitness, Variation, Herbivory

1. Introduction

Plants exhibit phenotypic plasticity and respond to variations in environmental conditions by acclimation. This is likely to have influenced the outcome of many studies, but is not often acknowledged in the literature. In most *Arabidopsis* experiments published to date, artificially constant growth conditions have been used, in stark contrast to the fluctuating natural conditions that plants have experienced (with accompanying selective pressures) over millions of years. From an evolutionary perspective, the genotypes with the highest Darwinian fitness are those that have adapted most successfully to these selection pressures, which typically show strong variations across all time scales. The fluctuations encountered may include, for example, sunflecks under canopies (1), in which incident light intensities can change by orders of magnitudes in seconds; strong diurnal fluctuations in temperature, light intensity, and

humidity; and seasonal changes that cause cycling between conditions that promote and inhibit plant growth. The ability to perform well under different conditions is a major contributor to plant fitness, and the contribution of phenotypic plasticity to the ability to grow, develop, and reproduce under non-optimal conditions is more likely to be relevant in natural conditions than in those encountered in the lab where *Arabidopsis* experiments are typically performed.

Future trends in plant sciences, and *Arabidopsis* research in particular, are likely to encompass the development of improved experimental procedures for studying the responses of genetically well-defined genotypes under field and semi-field conditions (2–4). The importance of such studies is only beginning to emerge, with plant physiologists and molecular biologists starting to perform relevant experiments. In one of our first ventures, we performed a simple experiment in which the seed set was compared between *Arabidopsis* mutants and wild-type plants grown in the field. From the results, we were able to quantify the contribution of a single gene to seed set, which in *Arabidopsis* is a reasonably accurate proxy of Darwinian fitness (5). A major outcome of this experiment was the demonstration that mutations that do not have clear phenotypic consequences under laboratory conditions can significantly influence performance in the field and that the ability to respond to environmental variation is important to plants. We have since performed field experiments with *Arabidopsis* mutants and transgenics to study seed production (5–8) and physiological processes (9) in conjunction with microarray and metabolomic analyses (10). Although not all *Arabidopsis* experiments are suited to field conditions, we believe that field studies can be highly informative. Indeed, many important questions cannot be properly addressed without examining the performance of organisms in natural or close to natural environments. Our aims here are to allow others to learn from our mistakes and provide some inspiration and guidelines for researchers who wish to address such questions but do not have experience in setting up field experiments.

2. Materials

2.1. Permit

Most, if not all, countries where research with transgenic plants is performed have a regulatory legislative framework for such activities. These regulations differ between countries. Even within Europe, where they are covered by European Union (EU) Directive 2001/18/EC (11), there is no consensus between countries. Here, we describe as an example how this is done in Sweden, where the Swedish Board of Agriculture (Jordbruksverket) handles the applications. Other countries within the EU have similar authorities, and the EU has an overall regulatory policy for the use of transgenic plants. The corresponding authorities in other major

countries include the United States Department of Agriculture (USDA)/APHIS (Animal and Plant Health Inspection Service) in the USA and Office of the Gene Technology Regulator (OGTR) in Australia. Researchers planning experiments with transgenic plants are advised to begin the approval process as early as possible since the process from application to permission is often slow.

For research purposes, permits to grow transgenic plants in the field within the EU are denoted “deliberate release” in contrast to “contained use” (in the lab) and market release. Acquisition of a permit involves the following steps:

1. The application procedure requires a full application in the national language, plus a shorter SNIF (Summary Notification Information Format for the release of genetically modified higher plants) to be completed in English since these documents are distributed to many authorities and organizations within the EU. More information is given in the Appendix to Directive 2001/18/EC. The current fee in Sweden is 47,500 SEK (~5,150 EUR, ~7,050 USD in October 2010) per application, with no more than two species and three gene constructs. All applications are scrutinized by national authorities and sent to corresponding authorities in the other EU countries that are allowed to comment.
2. A decision is taken within 120 days, but this could be delayed if additional information is required (see Note 1). One application may cover a large number of transgenic lines, which typically have to be described independently. The introduced DNA fragment is the element of most concern to the regulatory authorities; where there is a collection of insertional lines, these can be described similarly. For instance, we have a very useful permit that covers use of SALK and other T-DNA knockout lines of all genes coding for photosynthetic proteins, and this permit is also valid for genes that are not involved in the primary photosynthetic reaction.
3. Once a permit has been granted, it is necessary to provide:
 - (a) A map of the field site (in our case a plot covering about 10 m² in the experimental garden at Umeå University).
 - (b) A copy of the written instructions provided to the field personnel.
 - (c) A copy of the notification provided to the local authorities (a simple letter stating that a field experiment is to be performed according to a certain permit).
 - (d) A copy of an advertisement in a local newspaper notifying the public of the experiment.
 - (e) A fee to cover the cost of an inspection to ensure that the written protocols are being followed, in addition to the cost of the application.

2.2. Field

For our field studies, we selected a field site on the campus at Umeå University adjacent to the greenhouses, which could be easily accessed and maintained. The actual area required depends on the size of the planned experiment. The size of the area we chose is about 10 m² and is capable of accommodating up to about 1,800 *Arabidopsis* plants. Factors that need to be considered when selecting a field site are:

1. For management purposes, how frequently access will be required.
2. The physical environment of the site, for example, whether the plants are likely to be in shadow, the level of exposure to the wind and the sun, and the proximity of sexually compatible plants (in our case *Arabidopsis* or *Cardaminopsis*).
3. The security of the site (see Note 2).

2.3. Net Cage

The possibility of cross-pollination from wild plants can be reduced by using a net cage. The net should be chosen to allow as much light through as possible but prevent access by insects. The net cage can be constructed around a wooden frame built by stapling the net to 2-cm-diameter wooden sticks held together by metal corner plates.

Provision of a top lid for the cage enables the plants to be accessed and handled easily, for example, when reorganizing, taking pictures, or treating the plants against herbivores. The lid itself consists of a frame that fits exactly to the fence, laying across the top with the net hanging down the edges. We use cages that are two trays wide and five trays long; the optimal layout depends on the overall dimensions of the field site. We recommend a cage height of at least 50 cm to allow undisturbed growth of the inflorescence, see Fig. 1.

2.4. Seed Counting

If fitness is one of the parameters to be measured, a suitable place to count seeds must be arranged. This facility should also be described in the application for permission to handle GM plant material and seeds. A normal lab bench covered with plastic that can be easily disposed of afterwards is adequate. Good lighting is important in places where seed manipulation is performed as seeds are small so counting and sorting them can strain the eyes.

2.5. Other Materials

Basic items include:

1. Pots of a suitable size. The pot size will determine how much soil and hence nutrients are accessible to the plant. If soil is also placed underneath (and around) the pots, a size of 6 × cm 6 cm or 7 cm × 7 cm is appropriate (we use pots supplied by VEFI A/S, Norway).

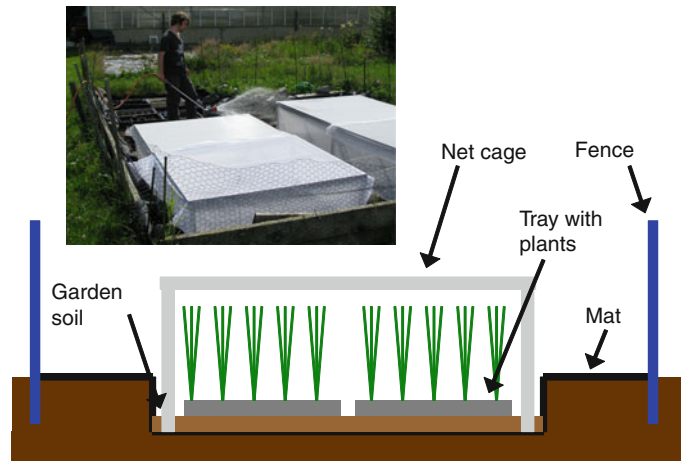


Fig. 1. Schematic illustration of the field site. The *inset* shows the field in Umeå with net cages covering the plants.

2. Soil is required for pots and the field site. We use “Yrkesplantjord” (Weibull Trädgård AB, Hammenhög, Sweden), a standard garden variety that has a good nutrient composition for our purposes.
3. Tags for marking each pot with a range of distinctive colours (see Note 3).
4. A light meter to measure the actual levels of light experienced by the plants.
5. A camera for documenting the growth and appearance of plants.
6. Sticks to tie plants to when the inflorescence is growing.
7. A permanent marker with ink that resists sun bleaching (see Note 3).

3. Methods

3.1. Plant Material

1. Vernalize seeds for 2 days in water at 4°C.
2. Prepare pots, sow the seeds, and germinate indoors to ensure even germination (seedlings are very small and fragile at this stage, see Note 4). In our studies, the main focus has been on the effects of mutations on plants' phenotypes and seed production, usually using individual plants in separate pots. However, where competition for resources is the main focus of the experiment, individuals could be planted together at this point.

3. Mark each pot/tray with a permanent marker. Different genotypes can be differentiated with differently coloured tags (see Note 3).
4. When seedlings appear (if several seeds have been sown in each pot), remove all but one from each pot.
5. Fill the trays with a 2-cm layer of soil. Place the pots on the soil and fill the space between the pots with soil.
6. If several genotypes are to be compared, place pots containing the different genotypes in random locations in each tray (see Note 5). The optimum number of pots per tray, that is, how close to grow them, depends on a trade-off between the size of the field site, convenience for the researcher, and the accessibility of nutrients for the plants, which can be seen in Fig. 2 (see Note 6).
7. Take care when transferring the prepared trays to the field site to minimize the risk of unintentional release of transgenic seeds. If they are being transferred to the field site in sunny weather, allow the seedlings to acclimate in the shade for a day before exposing them to full sunlight.
8. If the plants are allowed to flower, tie together the inflorescences to prevent interference with neighboring plants, which could complicate seed counting.
9. When the first siliques ripen, transfer the plants carefully to count seeds, harvest, or discard. When plants have set seeds,



Fig. 2. *Arabidopsis* plant performance on standard soil with different distances to neighboring plants. All plants were the same age, but those at position A grew close to each other (distance between pots, ca. 2 cm), those at position B had access to some additional soil, and those at position C had access to most of the soil in a whole tray.

handle them with great care. We use a large sealable cardboard box, covered on the inside with plastic, to transport the plants and trays from the field to the lab, where we count the seeds. The whole interior of the box is discarded with the remainder of the GM plant waste.

3.2. Preparation of the Field

In order to reduce the risk of unintentional release of transgenic material and to maximize the value of the results obtained from our experiments, we organize the field site as follows (Fig. 1):

1. At the site, remove 10–20 cm of the topsoil.
2. Cover the remaining soil by a plastic water-permeable mat normally used for weed control and keep in place with the help of stones. All of the material above the mat will, after completion of the experiment, be regarded as GM material and should be treated accordingly.
3. Spread a soil layer (we use a standard garden variety, “Yrkesplantjord” from Weibull Trädgård AB, Sweden) to a depth of about 5 cm on top of the mat. This establishes a more humid, and hopefully less artificial, environment for the plants.
4. Surround the whole site by a low fence to prevent it from being disturbed unintentionally.
5. Clear the area up to 10 m around the experimental site of all *Arabidopsis* and *Cardaminopsis* plants, which could hybridize with the experimental *Arabidopsis* (12) plants. This is a requirement in our permit, which also stipulates that the area should be regularly inspected and all *Arabidopsis* and *Cardaminopsis* plants found should be destroyed.
6. Place net cages over the site. Coverage with an insect net is a requirement in our permit to prevent unintentional insect-borne pollination (see Note 7). Before plants subsequently bolt, cover the whole site by an insect net, placed at least 50 cm above ground level, to allow the plants to grow undisturbed as seen in Fig. 1. One effect of the insect net is that it shades the plants (the net we use reduces the light intensity by almost 50%). However, as peak light intensities outdoors can approach $2,000 \mu\text{mol}/\text{m}^2/\text{s}$, much higher than those normally used in growth chambers, the rapid and unpredictable light variation that characterizes natural growing conditions is not affected significantly.
7. After the experiment, clean the field. Pack all transgenic material (i.e., everything above the mat) at the site in sealed double plastic bags and transport to the lab, where it is treated together with other transgenic waste according to local regulations.

8. During the next year, inspect the area for growing *Arabidopsis* plants and discard any found according to the GMO waste treatment protocol.

3.3. Field Maintenance

1. Check the site at least every second day and water if necessary. Our experience is that insect attacks can occur very suddenly. If herbivorous insects appear, treat them with insecticides, or if insect attack is a feature of interest in the experiment, record any relevant information. Collect any herbivores attacking the plants for later identification by specialists. Note any other unexpected events that occur (see Note 8).
2. Record the weather conditions (see Note 9). There may be a weather station located nearby and, if so, information from it can be supplemented with local records. Also record the minimum and maximum levels of incident light experienced by the plants and the reduction in light transmission caused by the cage net.
3. It is important to ensure that the net is covering the area before the plants bolt.

3.4. Seed Counting

A particular interest when growing *Arabidopsis* in the field could be to investigate the seed production of a specific genotype. When the first siliques are ripe, we normally bring the plants indoors and count the seeds. Our procedure is to:

1. Select five random/representative siliques.
2. Count all seeds from the five siliques.
3. Count all of the siliques of the whole plant.

To calculate total seed production, multiply the number of seeds per silique by the total number of siliques (Fig. 3). Germination tests can also be performed, but since seeds are typically collected on a single occasion (for practical reasons), it should be noted that many of the seeds, for example, those in pods that are not yet ripe, may not be mature.

Finally, it is essential to discard all of the plant materials, pots, and soil above the mat as GM material.

4. Notes

1. Apply for permission in time. Check with your authorities how long they expect the progress of an application to take and then start well in advance.
2. One potential concern is that planned experiments could be disrupted by activities of organizations or individuals opposed to work with transgenic plants. We notified the public about

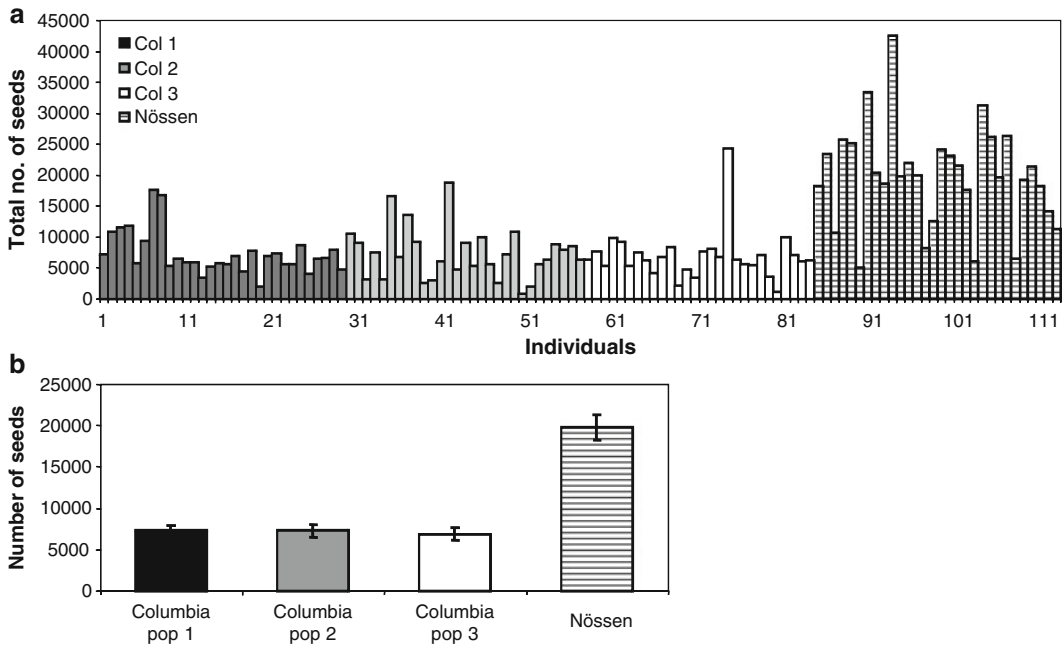


Fig. 3. Variation in seed set of plants representing two wild-type *Arabidopsis* lines, Columbia (obtained from three different laboratories) and Nössen, grown in the same experiment. Despite the high variation between individuals (a), mean values for all Columbia populations did not significantly differ (b); variation is shown as SE ($n=30$).

our experiments in newspaper advertisements and, on two occasions, in newspaper articles written by local journalists with an interest in the research. We have never experienced a negative public reaction to our experiments. We believe that the acceptance of transgenic field experiments depends on (1) the trait being modified and the plant species used, (2) local and national opinion among the public and politicians, and (3) the attitude of the public to the credibility of the organization performing the experiments. The species may be the least important factor, but in this respect, *Arabidopsis* is a good choice. Although regarded as a weed, it does not show invasive properties and is not a species of significance outside the scientific community, in contrast to plants such as tobacco, rice, or soybean, for instance. The credibility of the organization is, in our opinion, likely to be a major factor. For instance, a public body with governmental funding, such as a university, may have more credibility (depending on public attitudes) than a research center that receives some funding from private industry with vested interests in the outcome of the experiments, and some companies with poor safety records may have very little credibility.

3. Over a month or two, exposure to extremes of weather such as the sun and rain will affect the tags. The colour of the tag and

the text will eventually bleach, so use of strongly coloured tags and a good permanent marker for labeling will help avoid confusion about what was actually planted where and when. We also make a detailed map of the experimental site, showing the locations of individual plants.

4. *Arabidopsis* seedlings are very sensitive to drought. In the field, the seedlings are also exposed to wind, which causes water to evaporate more quickly than in still conditions. To prevent total loss of experimental material, plants need to be well nursed and watered during the first week in the field. We also closely follow the weather forecasts and start the experiments accordingly, avoiding planting out seedlings on very sunny, rainy, windy, or cold days.
5. Randomizing plant sample locations is essential since conditions even within a tray can vary significantly. We calculate how many pots of each genotype should be placed in each tray and then randomize them. Simple randomizing methods like throwing a dice are suitable for most normal-scale experiments, but more sophisticated methods may be required for larger experiments.
6. The amount of soil and plants in each tray can limit the plants' access to nutrients and strongly affect their growth. It is important to space the pots equal distances apart. Variation in the amounts of soil between trays is not as significant since it can be corrected statistically later (as between-tray differences). If soil is placed in the trays under and around pots, the plant roots will grow out of the pot and access nutrients from the adjacent environment. However, during the first few weeks in the field, pots can be lifted regularly to prevent plants rooting outside their own pots; this also helps to equalize nutrition levels (Fig. 2).
7. *Arabidopsis* is an almost obligate self-pollinator, but the rationale behind the preventative measures required by the authorities is that chewing insects could mediate cross-pollination if they mimicked laboratory procedures for making *Arabidopsis* crosses (i.e., removing petals, carpels, and stamens from closed flower buds that have not yet been pollinated while leaving the pistil intact). In practice, the possibility of a cross occurring unintentionally between two *Arabidopsis* genotypes is small, but this simple measure also provides better control over insect herbivory.
8. Variations in field conditions and the weather can cause differences in the average size of plants. In our field experiments, we have observed more than twofold differences in average plant size between years, with consequent effects on seed production, as illustrated in Fig. 4. There have also been a number of unexpected events, including heavy rain that left the plants

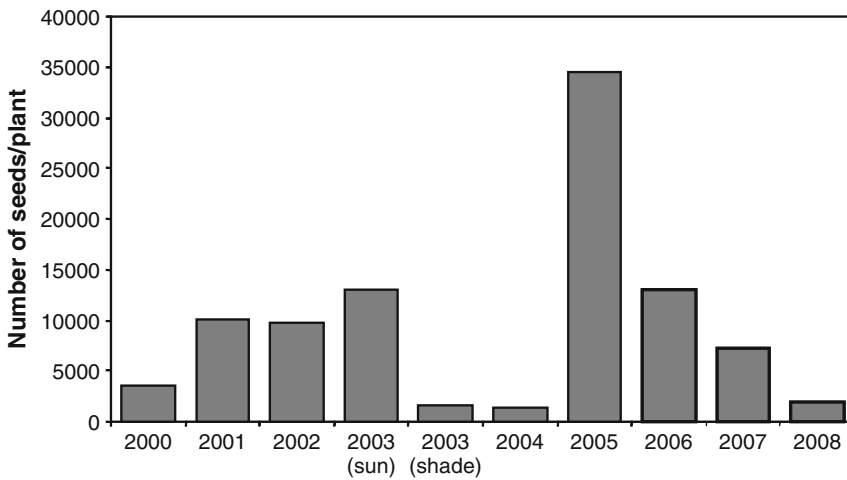


Fig. 4. Year-to-year variation in *Arabidopsis* mean seed production. All experiments except one batch from 2003 were performed in full sunlight. Note that there were also some differences in the experimental designs.

submerged in a pool of water, sudden attacks by herbivores, cold snaps that killed all of the seedlings, and even a bird flipping the marked tags.

9. Keep a detailed experimental diary. The recorded data may prove to be very valuable later, and take plenty of photos—you never know when you will need them! A shortage of appropriate documentation may be a source of regret when the data need to be interpreted.

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