P. Narayanasamy

Molecular Biology in Plant Pathogenesis and Disease Management:

Disease Management

Volume 3

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of My Parents
for their Love and Affection

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Preface

Studies on various aspects of plant-pathogen interactions have the primary goal of providing information that may be useful for developing effective crop disease management systems. Molecular techniques have accelerated the pace of developing short- and long-term strategies of disease management. The strategies that do not depend on host genome modification are based on the principles of exclusion and eradication of pathogens. Molecular methods have played significant role in precise detection, identification, differentiation and quantification of pathogens in symptomatic and asymptomatic plant tissues, resulting in prevention by plant quarantines of introduction of exotic pathogens and elimination of destructive pathogens in infected plants or planting materials by certification programs. Development of cultivars with built-in resistance to microbial pathogens is considered as the most plausible disease management strategy. This approach involves genome modification by incorporation of resistance gene(s) by conventional breeding methods or transformation of plants by incorporation of desired genes from diverse sources.

Molecular techniques have greatly promoted the understanding of the mechanisms employed by plants to defend themselves against different kinds of microbial pathogens. Molecular studies on R proteins and downstream signal networks have focused the attention on the possibility of using *R* genes more effectively for containing the diseases. Marker-assisted selection (MAS) procedure has been extensively employed to select rapidly genotypes with resistance to disease(s). Post-transcriptional gene silencing (PTGS) in plants has been shown to be an effective basis for studying disease resistance mechanisms operating in some pathosystems. PTGS is a potential RNA-mediated defense response capable of protecting plants against viral pathogens. It has been possible to monitor the expression of thousands of host/pathogen genes simultaneously under different defense-related treatments. A better understanding of the role of various genes or gene clusters in infection and resistance phenomena would be possible by applying DNA microarray technology. Genetic engineering has helped to introduce novel resistance genes from diverse sources into crop plants to protect them against the economically important pathogens. Strategy depending on induction of natural defense mechanisms by employing biotic and abiotic inducers of resistance has been shown to be a practical possibility in certain crops. Although use of chemicals for containing crop diseases is followed frequently, emergence of pathogen strains resistant to the chemicals

has become a serious problem to be overcome. Molecular techniques have been employed to identify and monitor the pathogen strains exhibiting resistance to chemicals. With the possibility of sequencing of whole genomes of plants and pathogens of economic importance, a sound basis may be available for developing effective disease management systems, resulting in safe environment, food and feed for the humans and other organisms existing in this planet earth.

This book presents updated and comprehensive information in an easily understandable style on the molecular biology of plant-pathogen interactions in three volumes: (1) Microbial plant pathogens, (2) Molecular biology of plant disease development and (3) Molecular biology in crop disease management. The usefulness and effectiveness of molecular techniques to establish the identity of pathogens precisely, to have a better understanding of the intricacies of the success or failure of pathogen infection respectively in compatible and incompatible plant species and to develop more effective disease management systems is highlighted with suitable examples. Appendices containing protocols included in appropriate chapters will be useful for students, teachers and researchers of various departments offering courses and pursuing research programs in molecular biology in general and plant pathology in particular.

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Chapter 1
Introduction

Various aspects of interactions between plants and microbial pathogens are studied with the primary aim of developing effective disease management systems based on the principles of exclusion, immunization and eradication, in order to reduce the qualitative and quantitative losses caused by microbial pathogens. The effectiveness of both short- and long-term strategies to contain the pathogen development in infected plants and to restrict the disease spread under field conditions has to be assessed. The usefulness of molecular methods for selection, adoption and integration of suitable disease management strategies to keep the pathogens at bay is discussed in six chapters included in the volume 3 of this treatise.

1.1 Strategies Not Depending on Genome Modification

The basic step in the development of an integrated disease management system is the use of seeds and planting materials certified to be free of designated pathogens and prevention of introduction of exotic pathogen(s) through imported plant materials that may or may not exhibit symptoms of infection. Domestic and international plant quarantines and certification programs need techniques that can provide reliable results rapidly. Several molecular techniques can be employed for detection, identification, differentiation and quantification of targeted microbial pathogen(s) to meet the stringent requirements of quarantines and certification programs. Different kinds of certification programs are in operation in various countries to suit their requirements, resulting in the elimination of infected plants and planting materials ensuring the supply of disease-free planting materials to the growers (Pallás et al. 2000; Narayanasamy 2001).

As an alternative strategy to chemical application for disease control, utilization of biocontrol agents (BCAs) holds promise because of its ecofriendly nature. Due to significant variations in the biocontrol potential of the fungal or bacterial species that can be employed as BCAs, precise identification of the strains/isolates, quantification and monitoring the population levels of the introduced BCA strains at different periods by using molecular techniques, become essential as in *Aureobasidium pullulans* (Schena et al. 1999, 2002). Molecular markers have been employed

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for identification and characterization of strains of *Bacillus subtilis* effective against soilborne pathogens such as *Rhizoctonia solani* and *Pythium ultimum* (Joshi and McSpadden Gardener 2006).

1.2 Strategies Depending on Genome Modification

Development of cultivars with built-in resistance to crop diseases is acknowledged to be the most desirable disease management strategy. It is ecofriendly and does not demand generally any additional effort other than normal cultivation practices adopted by the growers. Enhancement of host resistance to microbial infection may be achieved by (i) incorporating resistance (*R*) genes from cultivars or wild relatives through conventional breeding methods, (ii) transforming plants to express genes of choice from plants or other biological sources and (iii) inducing natural disease resistance of plants by applying biotic or abiotic inducers of resistance.

Understanding the mechanisms employed by plants to defend themselves against fungal, bacterial and viral pathogens may be useful to develop novel strategies to increase the level of resistance to diseases in susceptible cultivars. The *R* genes have been employed in resistance breeding programs with varying degrees of success. Cultivars with resistance to diseases can be developed much earlier by adopting marker-assisted selection (MAS) procedure compared to the traditional breeding methods. The molecular research on R proteins and downstream signal transduction networks has indicated the possibility of using *R* genes more effectively for disease control. Several signal transduction components in the defense networks have been characterized and they are being exploited as switches by which resistance can be activated against a range of pathogens (McDowell and Woffenden 2003). Evidence for allele-specific interaction between alleles of a particular R protein and corresponding pathogen-derived Avr protein has been obtained. In contrast, Avr proteins can function also as effectors promoting pathogen virulence in susceptible plant species incapable of recognizing the pathogen-associated molecular patterns (PAMPs).

Plant pathogens have evolved mechanisms independently to deliver effectors into plant cell cytoplasm. Cloning of *R* gene was achieved for the first time, by transposon tagging of *Hm1*, a gene in maize that governs resistance to race 1 strain of *Cochliobolus carbonum*. The gene encodes a reductase that inactivates the potent host-specific toxin (HST) elaborated by *H. carabonum* (Johal and Briggs 1992). Later successful cloning of the *Pto* gene that confers resistance to tomato against *Pseudomonas syringae* pv. *tomato* (*Pst*) was reported (Martin et al. 1993). This *avr*-induced resistance was shown to be due to a protein with similarity to serine-threonine protein kinases. In these cases, *R* genes appeared to function as receptors for *avr* gene products of pathogens. Detection of an effector by an R protein triggers rapid activation of very effective defense responses (Sequeira 2000; Dangl and McDowell 2006). The defense responses may be of two types namely non-host resistance effective against all races of the pathogen and host resistance effective against only some races of the pathogen. However, several components of

the signaling pathways appear to be common to both types of resistance (Thordal-Christensen 2003).

The emergence of *Arabidopsis thaliana* as a model plant has been responsible for accumulation of significant amount information in different branches of biological sciences in general. As the genome is comparatively small in size and entirely sequenced, *A. thaliana* is being used as a basic reference for all studies related to disease development and resistance. However, the need for verifying the relevance of the data obtained using *Arabidopsis* to understand the molecular basis of interaction of pathogens with economically important crops, has been well realized. Post-transcriptional gene silencing (PTGS) in plants, an RNA-degradation machinery, has been shown to be an effective basis for studying disease resistance mechanism in certain pathosystems. There is a complex relationship between PTGS and virus infection/ resistance. PTGS in plants inactivates some aberrant or highly expressed RNAs in a sequence-specific manner in the host cell cytoplasm and it is an innate antiviral defense in plants and animals (Soosar et al. 2005). As the ds-RNA is not synthesized naturally in plant cell cytoplasm, the plant's resistance mechanism reacts to the presence ds-RNA produced during virus replication. Virus-induced gene silencing (VIGS) is a characteristic manifestation of PTGS in which viruses are both triggers and targets of silencing. PTGS has the potential to be an RNA-mediated defense response to protect plants against plant viruses (Moissiard and Voinnet 2004; Vaucheret et al. 2001).

Endogenous small interfering RNAs (siRNAs) and microRNAs (miRNAs) have been shown to be important regulators of eukaryotic gene expression by guiding mRNA cleavage, translation inhibition or chromatin modification. The significant role of miRNA in basal defense against *Pseudomonas syringae* by regulating auxin signaling was demonstrated by Navarro et al. (2006). It has been possible to monitor the expression of thousands of genes simultaneously under different defense-related treatments and over different points of time, with the advent of large scale genomic sequencing, expressed sequence tagging and DNA microarray techniques. New pathogenesis-related genes, coregulated genes and associated regulatory system have been identified and characterized. DNA microarrays have been applied to study plant-pathogen interactions and downstream defense signaling providing a better understanding of the role of various genes or gene clusters in infection and resistance phenomena (Katiyar-Agarwal et al. 2006; Abramovitch et al. 2006).

The imperative need for alternative approaches to overcome the obstacles associated with conventional breeding methods was realized by researchers in time. The development of plant genetic transformation technology has provided a powerful tool to transfer desired genes from diverse sources to obtain plants with resistance to crop diseases. Genetic engineering methods enable the researchers to introduce novel resistance genes including genes from sexually incompatible species. Further, synthetic genes can also be designed to interfere with specific pathogens or virulence factors. *Agrobacterium tumefaciens*-mediated transformation protocols have provided significant success in transferring genes from diverse sources to confer resistance to diseases. Crops expressing the coat protein genes of viruses have shown encouraging results in terms of yield and quality of produce. Transgenic papaya

lines expressing the coat protein (CP) gene of *Papaya ringspot virus* (PRSV) have reached the stage for commercial exploitation (Souza Jr et al. 2005). The possibility of tackling the *Fusarium* wilt disease of tomato by developing transgenic plants expressing glucanase and chitinase genes was indicated by Ouyang et al. (2005). The usefulness of employing the genes expressing polygalacturonase-inhibiting proteins (PGIPs) for protecting tomato against *Botrytis cinerea* causing grey mold disease was indicated by Powell et al. (2000). A novel method of enhancing resistance of pears to the fire blight disease caused by *Erwinia amylovora* by transforming the pear plants with the elicitor gene $hrpN_{ea}$ was shown to be a feasible approach for reducing losses due to this disease (Malnoy et al. 2005).

1.3 Strategies Depending on Induction of Natural Defense Mechanisms

Two principal types of molecular mechanisms are known to be involved in the activation of natural disease resistance (NDR) systems existing in plants, when biotic or abiotic inducers are applied. Systemic acquired resistance (SAR) develops locally or systemically in response to pathogen infection or treatment with inducers of disease resistance. SAR is mediated by salicylic acid (SA)-dependent process, whereas induced systemic resistance (ISR) develops as a result of colonization of plant roots by plant growth-promoting rhizobacteria (PGPR) and it is mediated by jasmonate or ethylene-sensitive pathway (Pieterse et al. 1998). Development of resistance locally in treated tissues and systemically in tissues or organs far away from the site of application has been demonstrated. The effectiveness of SAR and ISR against fungal, bacterial and viral diseases to different degrees has been reported, suggesting the feasibility of adopting this approach for disease control in certain crops. The molecular mechanisms operating during induction of resistance in *A. thaliana*, form a window view of the interplay between microbial pathogens and other plant species treated with inducers (Wang et al. 2005). *Pythium oligandrum*, a biocontrol agent, or its elicitin oligandrin is able to induce the expression of defense-related genes involved in the production of lytic enzymes and consequently the level of resistance of grapevine plants to *B. cinerea* is significantly enhanced (Mohamed et al. 2007).

1.4 Strategies Based on Direct Effects of Chemicals on Pathogens

Various chemicals are applied on crops to restrict the incidence and spread of diseases. Although the chemicals are able to provide effective control of the target pathogen(s), the danger due to emergence of strains of pathogens showing resistance to chemicals that have specific sites of action on the pathogen, has been well realized. The changes in the nucleotide sequences of the β-tubulin gene of fungal pathogens have been revealed by molecular techniques. Application of molecular technique(s) to detect the fungicide resistant strains and subsequent development

of resistance management procedure has been shown to be an effective strategy for making right decisions in crop production (Reimann and Deising 2005).

The molecular techniques have the potential to be more precise, rapid, reliable and reproducible compared with the conventional techniques depending on pathogen isolation in cultures and microscopical observations. In addition, the molecular methods are amenable for automation making it possible to handle large amounts of experimental materials. With the possibility of genomics, proteomics and metabolomics techniques becoming available for many pathogens and major crop plant species, it would be possible to understand the interactions of plants with pathogens more comprehensively. Consequently a sound basis may be available for working out disease management systems for combating the pathogens at vulnerable stages in their life cycle, so that crops may be protected more effectively.

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Chapter 2
Exclusion and Elimination of Microbial Plant Pathogens

Abstract Following globalization, enormous increase in the movement of passengers and cargo shipments has become unavoidable. This situation has also increased significantly the possibility for introduction of new pathogens or new strains of the existing pathogens, necessitating application of diagnostic techniques that have the potential to provide reliable results rapidly. Identification of the pathogens precisely is essential to exclude the more virulent strains or pathogens into the given geographical location or country. For this purpose, the application of molecular techniques has been shown to be effective and advantageous. The detection of pathogens in seeds and propagative plant materials has helped eliminate infected consignments. The suitability and effectiveness of the molecular methods for detection of microbial pathogen is discussed with appropriate case studies.

Modern agricultural practices, globalization of trade and large scale movement of people and goods have created conditions favorable for introduction, incidence and spread of plant diseases caused by microbial pathogens. Crop management systems based on various principles aim (i) to reduce the introduction of the pathogen/disease; (ii) to suppress the initial amount of inoculum and (iii) to improve the level of resistance of crop cultivars to disease(s). Establishment of domestic and international plant quarantines and production of disease-free seeds and propagative materials have been significantly effective in preventing/reducing the disease incidence of various diseases caused by microbial pathogens.

With significant improvements made in passenger traffic and cargo transshipments via air and sea, the probability of unintentional introduction of pathogens has also increased by many folds. Natural introductions of invasive plant pathogens and insect pests have been estimated to be responsible for more than ten billion dollars annually in the United States alone (Pimentel et al. 2000). Regulatory methods have been formulated with the aim of preventing the import and spread of plant pathogens into the country, state or province. Legislative measures are formulated to regulate cultivation of crops and distribution of propagative materials between countries or states within the country. Regulatory control is enforced by establishment of quarantines and inspection of crops in field/greenhouses/warehouses for certification of produce to indicate the health status of the agricultural produce. Introduction of

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certain invasive pathogens has led to development of high-impact epidemics accounting for massive economic loss and sociological upheaval (Kingslover et al. 1983; Campbell et al. 1999). In addition, the perceived threat of intentional introduction with a potential to cause considerable damage to the agricultural and natural systems appears to be of great concern for some countries. Furthermore, the formation of new races and biotypes of indigenous pathogens adds another dimension to the problem of formulating effective systems to keep the pathogens at bay. A plant biosecurity system with the capability for early detection, accurate diagnosis and rapid response is required to prevent the establishment and dispersal of pathogens after introduction and to minimize the adverse effects of such introduced and newly evolved pathogens or races or biotypes (Stack et al. 2006).

2.1 Exclusion of Microbial Plant Pathogens

The plant quarantines, established with the primary objective of preventing the introduction and spread of diseases into new areas/countries, helps protect agriculture and the environment from avoidable damage to crops. The importance of establishing well-equipped quarantines has been recognized, after adoption of the General Agreement on Tariffs and Trade (GATT), as there is a dramatic increase in the movement of plant products, necessitating the enforcement of sanitary and phytosanitary measures at the global level. The International Plant Protection Convention (IPPC) was established in 1991 following the acceptance of GATT by the majority of countries. Basic principles required for formulating standards for plant quarantine procedures in relation to the international trade by an expert committee have been laid down (FAO 1991). The principles of establishing plant quarantines recognize the sovereignty of the country which has the right to implement the phytosanitary measures deemed fit by that country. An organism is considered to be of quarantine significance (QS), if its exclusion is perceived as important enough to agriculture and natural vegetation of the importing country.

2.1.1 Seeds and Propagative Plant Materials

The infected seeds and asexually propagated plant materials such as tubers, bulbs and setts are the primary sources of infection. The populations of microbial pathogens – fungi, bacteria and viruses – present in the seeds and propagative planting materials have to be determined, based on the assessment of levels of infection using conventional and/or molecular detection and quantification methods. The advantages of employing molecular methods over conventional procedures have been discussed in Volume 1 Chapter 2. The tolerance limits for various pathogens have been prescribed by the International Seed Testing Association (ISTA). Most of the countries enforce zero tolerance to prevent the introduction of new pathogens into those countries. The possibility of introduction of fungal diseases such as celery leaf spot (*Septoria*

apicola), carrot leaf blight (*Alternaria dauci*) and onion neck rot (*Botrytis allii*), bacterial disease like bean halo blight (*Pseudomonas syringae* pv. *phaseolicola*) and virus diseases such as lettuce mosaic, soybean mosaic and bean common mosaic diseases through seeds has been recognized. Production of disease-free seeds to prevent the introduction of the causative agents into other countries has been strongly emphasized (Agarwal and Sinclair 1996; Maude 1996; Narayanasamy 2002). The International Seed Health Initiative (ISHI) founded in 1993 is an international consortium of seed industry and plant pathologists involved in seed health testing. Development of efficient, reliable seed health testing protocols in a timely manner is the primary objective of ISHI to assure that seed lots are sufficiently healthy for world-wide movement and to have a means of quickly testing new technologies for incorporation into seed health testing protocols (Maddox 1998).

Several techniques for the detection, identification, differentiation and quantification of microbial plant pathogens are available. The methods suitable for application in plant quarantines should have the following criteria: (i) results obtained should be reliable with high specificity; (ii) results should be available rapidly; (iii) it should be possible to assess pathogen population in question in relation to other pathogen(s); (iv) the technique capable of detecting two or more pathogens may be preferable; (v) the technique should be very sensitive, capable of detecting the pathogen(s) present in low concentration; (vi) it should be possible to detect latent/quiescent infections in plants, fruits or vegetables and (vii) the technique that can detect qualitatively and determine quantitatively the mycotoxins present in the seeds, fruits and vegetables may be preferable.

A serious threat to the export market for wheat from US to other countries was through seed infection by Karnal bunt disease. The available PCR assay could not differentiate *Tilletia indica* causing Karnal bunt disease from *T*. *walkeri* infecting rye grass. By employing five sets of PCR primers specific to *T*. *indica*, it could be precisely detected in wheat samples, enabling rapid identification and differentiation of the pathogen (Frederick et al. 2000). Carrot seeds are infected by *Alternaria alternata*, *A*. *radicina* and *A*. *dauci*, the former two species possessing high toxigenic potential. A PCR assay employing species-specific primers based on sequences of the ITS regions of the ribosomal repeat (rDNA) was useful for the differentiation of the three *Alternaria* species on carrot seeds and roots. The PCR assay can be used preferably, if results are required rapidly (Konstantinova et al. 2002). Use of disease-free seeds of crucifers is considered to be the effective management strategy for black spot disease of crucifers caused by *A*. *brassica*. A real-time PCR using primers designed on the basis of the sequence of two clustered genes potentially involved in pathogenicity. *A*. *brassicae* was specifically detected in the DNA extracted from seeds (Guillemette et al. 2004).

Detection of bacterial pathogens in seeds can be made more reliable by incorporating a biological or immunological step prior to conventional PCR. The bacteria present in the seeds are isolated in a general agar medium by plating the aqueous extract of the seeds and incubated for 45–48 h. The harvested bacterial cells are subjected to enzymatic amplification of DNA sequences of target bacteria. This technique BIO-PCR can detect *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) even if one bean seed

in a lot of 400–600 seeds, is infected (Mosqueda-Cano and Herrera-Estrella 1997). Immuno-magnetic separation (IMS) using specific antisera to concentrate *Acidovorax avenae* subsp. *citrulli* present in watermelon seeds, followed by PCR assay was shown to improve the sensitivity and specificity of the diagnostic test. The combination assay IMS-PCR has greater sensitivity (100-folds), compared to conventional PCR assay and as low as 0.1% seed infection (1 in 1000) can be determined by this procedure (Walcott and Gitaitis 2000).

Infected seeds form the most important sources of virus infection, since the viruses can easily spread to new areas or other countries through infected seeds. The incidence of *High plains virus* (HPV) infecting maize has been recently observed in the US and its occurrence has been reported from several other countries. Hence, a serious threat to the export of maize to other countries was evident. Sweet corn plants raised from seeds imported from the US were tested in a quarantine level 3 glasshouses in New Zealand. Application of ELISA test and RT-PCR assay confirmed the presence of HPV. These experiments confirmed the seed transmission of HPV in maize seeds and emphasized the need for indexing the seeds in post-entry quarantines (PEQs) to prevent the introduction of new viruses. A procedure for inspecting plants and testing cereal seedlings in quarantines using RT-PCR assay was also developed (Lebas et al. 2005). In the case of *Erwinia stewartii* (*Pantoea stewartii*) causing Stewart's wilt disease, maize seeds from the US are prohibited by many countries to prevent the introduction of this bacterial disease. The seed health test based on ELISA was prescribed by the National Seed Health System as the standard method for phytosanitary testing for the detection of *E. stewartii* (Pataky et al. 2004).

Immunoassays have been demonstrated to be useful for detection and quantification of microbial pathogens infecting propagative plant materials. The presence of *Spongospora subterranea* could be detected in potato tuber extract by using the polyclonal antibodies generated against the homogenate of spore balls (cystosori). The detection limit of ELISA was found to be as little as 0.08 sporeballs equivalent/ml (Harrison et al. 1993). Likewise, by using DPEM medium for anaerobic amplification of *Erwinia chrysanthemi*, ELISA test was used to detect the bacterial pathogen in seed potatoes. This procedure could be used for large scale application for detection of the pathogen in seed tubers and also for prediction of disease outbreaks in Switzerland (Cazelles et al. 1995). ELISA was shown to be as efficient as PCR assays in detecting *Clavibacter michiganensis* subsp. *michiganensis* (*Cms*), (causing potato brown rot disease) in symptomless potato tubers by efficient enrichment followed by DAS-ELISA test (Slack et al. 1996). Specific monoclonal antibodies that did not react with any of the 174 isolates of other pathogenic or unidentified bacteria isolated from potato tubers were used for this assay which had high level of specificity, with a detection limit of 1–10 CFU of *R. solanacearum* per ml (Caruso et al. 2002).

Spongospora subterranea could be detected in potato peel and tuber washings by employing specific primers (Sps1 and Sps2) based on sequences of the ITS region of rDNA of the target pathogen. These primers amplified a 391-bp product only from *S. subterranea*, but not from other fungi associated with potato tubers indicating the specificity of detection of the target pathogen. This procedure has the potential for application for disease risk assessment of seed potato stocks (Bell et al. 1999).

Potatoes are infected by more than 25 viruses causing serious losses (Salazar 1996). Among several diagnostic methods, PCR, RT-PCR and serological assays (DAS-ELISA) have been predominantly used for diagnosis of potato virus diseases. However, most of these techniques could detect only single virus. Multiplex RT-PCR assay has the potential for accommodating several primer pairs in one reaction, saving time and expense, in addition to its capacity for testing large number of samples. A multiplex RT-PCR system for simultaneous detection of five potato viruses using 18S rRNA as an internal control was developed. This new technique amplified cDNAs simultaneously from *Potato virus A* (PVA), *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV), *Potato virus S* (PVS) and *Potato virus X* (*PVX*), in addition to host 18S rRNA. This multiplex RT-PCR assay detected all viruses in different combinations and it was more sensitive (100-fold) for detection of PVX compared to commercially available DAS-ELISA protocol. PVX could be detected in some samples that DAS-ELISA failed to detect the virus (Du et al. 2006).

The infection of potato seed tubers by *Potato mop top virus* (PMTV) in seed tuber lots and ware potato was found to be significant in the US and Canada. The RT-PCR technique targeting CP gene in RNA3 of PMTV was highly efficient in detecting the virus (Xu et al. 2004). Diagnostic techniques that can provide results rapidly and reliably are needed for the detection of *Potato yellow vein virus* (PYVV), a quarantine pathogen to prevent its introduction or its subsequent spread in European and Mediterranean Plant Protection Organization (EPPO) region. Real-time RT-PCR assay based on TaqMan® chemistry or conventional PCR test was recommended for detection of PYVV reliably for enforcing quarantine regulations. In addition, these tests were also suggested for routine indexing of potato tubers for the presence of PYVV for production of virus-free seed tubers in South American countries where the incidence of this virus is quite high (López et al. 2006).

The imperative need to develop a reliable and sensitive technique providing results rapidly was found to be essential for the South African exporters to retain their competitive edge in the European market and access new markets like the United States. It is of quarantine importance to differentiate *Guignardia citricarpa* causing citrus black spot (CBS) disease from the harmless endophyte *G. mangiferae* which in not restricted by quarantine regulations. Timeliness and accuracy of pathogen detection and identification are critical factors for the export of citrus fruits, since the value of the consignment decreases rapidly with each additional day spent on holding. Hence, a same-day test that can provide results in one day was considered necessary for citrus fruit exports which were often rejected at harbor due to the presence of a single fruit spot suspected to be due to CBS disease. The one-day sensitive method involves the isolation of DNA directly from fruit lesions by means of the DNeasy Plant Minikit (Qiagen) and use of the primer set C1TR1C1 and CAMEL2 in conjunction with ITS4 primer to yield PCR amplicons of approximately 580-bp and 430-bp for *G. citricarpa* and *G. mangiferae* respectively. These two fungi could be distinguished unequivocally using this PCR protocol, eliminating the prior need for culturing these slow growing fungi, thereby shortening the time required to just one day to test for and verify the presence or absence of *G. citricarpa* in export consignments (Meyer et al. 2006).

2.1.2 Whole Plants

Colletotrichum acutatum can infect many crop plants including strawberry in which economic losses due to the pathogen are frequently high. As the incidence of the disease was absent in the Czech Republic, it was included in the List of Quarantine Pests to prevent its introduction. Three immunoassays namely plate-trapped antibody (PTA)-ELISA, immunoblot and immunofluorescence tests were employed for the detection of *C. acutatum* in extracts from petioles and roots of inoculated plants. Four polyclonal and two monoclonal antibodies were used. All antisera were genus-specific, but only one polyclonal antiserum IgG K91 showed high sensitivity. Using PTA-ELISA protocol and dot-blot, no cross-reaction with other fungi pathogenic to strawberry was observed. PTA-ELISA tests detected the pathogen in extracts of roots and crown of all cultivars at 7 dai, when no symptom of infection was visible. In petioles the infection was detected only in one cultivar, Elsanta. Dot-blot results were similar to that of PTA-ELISA test (Figs. 2.1 and 2.2). Latent infection of strawberry was also detected by these immunoassays. However, use of at least two of the tests is recommended for detecting latent infections in strawberry fruits (Krátká et al. 2002).

Infection of grapevine plants by *Xyllela fastidiosa* (*Xf*) has to be detected in the asymptomatic plants to prevent the spread of the disease. ELISA format was applied for the detection of *X. fastidiosa* in whole tissue samples and xylem fluid samples. Testing the xylem fluids by ELISA was more efficient than the tests on whole tissues from aymptomatic grapevine plants. There was no significant difference, when the

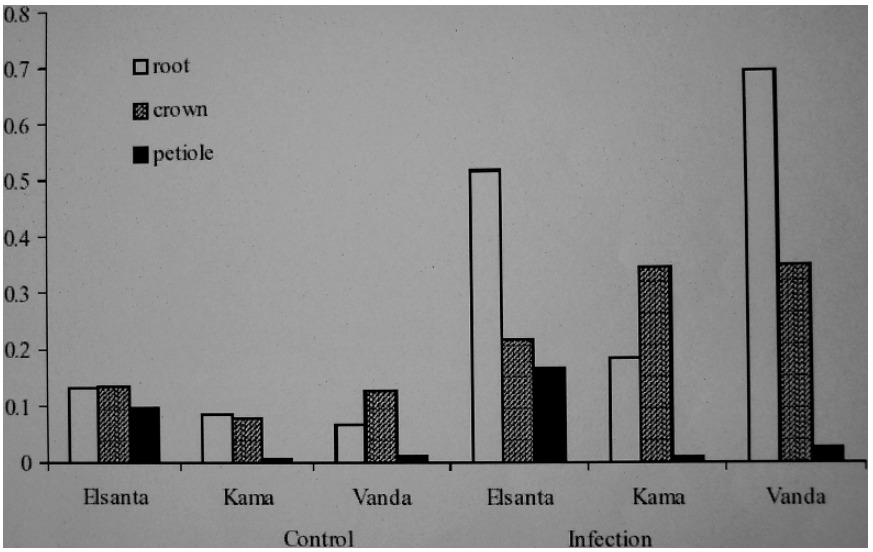

Fig. 2.1 Detection of *Colletotrichum acutatum* (isolate 12A) using PTA-ELISA in strawberry plants cvs. Elsanta, Kama and Vanda at 7 days after inoculation (dai) (Courtesy of Krátká et al. 2002; Plant Protection Science Institute, Praha, Czech Republic)

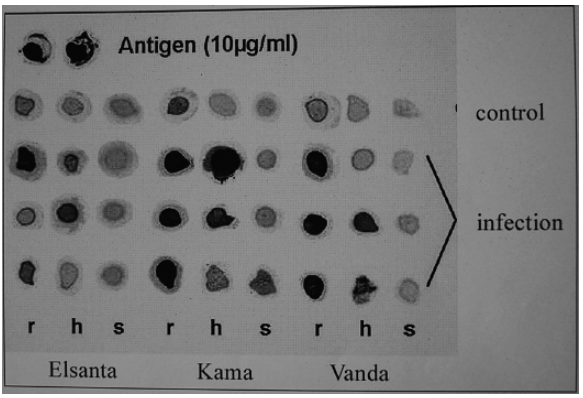

Fig. 2.2 Detection of *Colletotrichum acutatum* (isolate 30A) using dot-blot in strawberry plants cvs. Elsanta, Kama and Vanda at 7 days after inoculation (dai) r: Root; h: Crown; s: Petiole. (Courtesy of Krátká et al. 2002; Plant Protection Science Institute, Praha, Czech Republic)

frequencies of detection of pathogen by ELISA and PCR in the case of symptomatic grapevine plants were compared (Bextine and Miller 2004).

A major limitation for large scale application of molecular techniques for detection, identification and differentiation of microbial plant pathogens are trained personnel, well-equipped laboratories and cost-effectiveness of tests chosen. In addition, quarantine restrictions on carrying living organisms across the borders, prevent the use of equipped laboratories in other countries by developing nations. Nevertheless, it is possible to undertake pathogen isolation and purification in the countries that lack facilities for testing. The DNA of the pathogen to be investigated, can be sent to laboratories in other countries for analysis. Thus the bio-risks associated with moving the living organisms across the borders can be avoided. The lack of an easy DNA extraction procedure without using toxic organic compounds such as phenol and chloroform necessitated the development of a method for DNA of high quality and purity that is suitable for restriction digestion and PCR-based analysis. A protocol involving inactivation of proteins by using SDS/proteinase K and precipitating polysaccharides in the presence of high salt was developed for extracting plant, fungal and bacterial DNA of high quality. As many as 100 samples can be processed per day. The DNA isolated was entirely digested with five restriction enzymes: *Eco*RI, *Rsa*1, *Taq*1. *Eco*RV and *Hind*III. PCR analysis could be performed using enterobacterial repetitive intergenic consensus (ERIC) sequence, sequence characterized amplified region (SCAR) and random amplified microsatellite primers. The fungal pathogens such as *Colletotrichum lindemuthianum* and *Phaeoisariopsis griseola* and the bacterial pathogen *Xanthomonas campestris* pv. *phaseoli* infecting bean were isolated and their DNAs were subjected to PCR analysis for characterizing them. This newly developed procedure has the potential for application in quarantine services and marker-assisted selection (MAS) breeding (Mahuku 2004).

Strawberry plants are infected by several viruses which are transmitted by diverse types of vectors such as aphids, whiteflies, nematodes and fungi. Nucleic acid-based RT-PCR assay has been developed for the detection of most of the strawberry viruses. RT-PCR and real-time RT-PCR assays have been found to be effective for the detection of *Strawberry crinkle virus* (SCV) (Posthuma et al. 2002; Mumford et al. 2004).

Application of RT-PCR and ELISA tests for the detection of *Strawberry mild yellow edge virus* (SMYEV) was reported to be effective. These assays could detect SMYEV not only in strawberry, but also in all other sources of the virus characterized by symptoms on indicator plants (Thompson et al. 2003). *Strawberry mottle virus* (StMoV) was efficiently detected by employing primers based on conserved nucleotide sequence in the 3′ noncoding region. Sixteen isolates of StMoV were detected using a single primary pair in RT-PCR format (Thompson and Jelkmann 2003). The incidence of a new virus infecting strawberry designated *Strawberry chlorotic fleck virus* (StCFV) was detected by RT-PCR assay in commercial fields (Martin and Tzanetakis 2006).

The rapidity with which the diagnostic procedure provides the results is a critical factor for its application, even if the test has other advantages. For example, direct tissue blot immunoassay (DTBIA) has been shown to be a reliable and sensitive test for detection of *Citrus tristeza virus* (CTV), its sensitivity being comparable to RT-PCR assay (Lin et al. 2002). But this procedure required longer time (3–7 h) to give results. Hence, an improved DTBIA protocol that could provide results much earlier (within 1 h) was developed. Prints of fresh young stems of citrus plants (infected by CTV and healthy) were prepared by gently and evenly pressing the freshly cut surface of the stems onto nitrocellulose membrane. The blots of samples were incubated with prereaction solution of CTV-specific antibodies and labeled secondary antibodies [Appendix]. All samples from greenhouse plants infected by CTV (isolate T-36) were positive to CTV-specific PABs and MABs, whereas healthy plants were negative to all of the antibodies tested. The improved DTBIA was as reliable as the other immunoassays and almost as reliable as PCR in detecting CTV in field samples. The prereaction step introduced in the DTBIA protocol was responsible for the drastic reduction in the time required for obtaining the results (Lin et al. 2006).

Plant viruses, except a few are disseminated from infected plants to healthy plants by insects, nematodes and fungi that act as vectors. The viruses that have biological relationship with the vector species, are able to multiply in the insects and pass onto next generation through eggs. The vector insects are considered as important sources of infection for these propagative type of viruses. *Frankliniella occidentalis*, a thrip species is involved in the transmission of *Tomato spotted wilt virus* (TSWV) belonging to the genus *Tospovirus*, family *Bunyaviridae*. The Western flower thrips is a major pest of several agricultural and horticultural crops and it is a quarantine pest in Taiwan. For the efficient and reliable detection of *F. occidentalis*, a species-specific one-tube nested PCR-RFLP technique was developed. This method consisted of amplification of the rDNA region by a common primer pair CS 249/CS 250, followed by a second PCR with species-specific pair FO1/FO2 for *F. occidentalis*. The limit of detection was 1 pg DNA of *F. occidentalis* for this assay which is rapid and simple for the identification of the insect which is a major pest as well as a vector of an economically important virus that has a wide host range (Liu 2004).

Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) and bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) are the diseases that infect tomato. The symptoms induced by these bacterial pathogens are quite similar and likely to be confused with each other. In order to detect and identify these using crude DNA extracts and primer sets COR 1/2 (bacterial speck) and BSX 1/2 (bacterial

spot) was developed. All 29 pathogenic strains of *Pst* produced a 689-bp amplicon with COR 1/2, whereas the 37 geographically diverse *Xav* strains generated the 579-bp BSX 1/2 amplicon. The detection limit of the assays was 30–50 CFU/ reaction. Latent infections of apparently healthy and greenhouse-grown seedlings or young field plants may function as important sources of infection of the bacterial speck and bacterial spot diseases. The PCR protocol was modified to one where freeze-boil DNA extraction was applied to bacteria collected by centrifugation from the wash water from 10-g samples of symptomless young seedlings. The population of bacteria required for detection was 10^5 CFU of *Pst* (Cupples et al. 2006).

The choice of detection technique may be critically important in determining the success or failure of regulatory systems involved in preventing the introduction and spread of pathogen(s). A 5′ fluorogenic exonuclease (TaqMan) assay was developed to detect and quantify the fungal pathogen *Phytophthora ramorum* in plant materials. This method is sensitive being able to detect as little as 15 fg of target DNA, when used in nested design or 50 fg, when used in a single round of PCR. None of the other *Phytophthora* species (17) DNA was amplified by the primers employed, indicating high specificity of the test (Hayden et al. 2006).

2.2 Use of Disease-Free Planting Materials

Certification is a procedure that facilitates building up nursery stocks and also commercial production by subjecting them to controls for securing trueness-to-type and ensuring freedom from specified plant pathogens as directed by official regulations or endorsed by competent governmental agencies (Martelli and Walter 1998). The practical application of such conceptually simple measures can be expected to be the most powerful means for sanitary upgrading of the commercial production agencies involved in production of horticultural produce/plants. Nevertheless, little attention has been bestowed to promote internationally recognized certification schemes that following application, would enhance free trading of high quality nursery materials among the participating countries. Various political, commercial and technical impediments hamper the acceptance of international agreement on certification protocols (Rowhani et al. 2005).

The primary objective of certification schemes worldwide is to identify healthy sources for propagation through application of time-tested indexing procedures as well as modern molecular methods. The actual technique(s) employed may vary depending on the specific pathogen(s) targeted, the endemic disease(s) in the geographical location (country), availability of techniques, cost of testing and the requirements of the industries served. The first basic step is the establishment of foundation or nucellar source plants which are free from all known harmful pathogens and professionally identified for true-to-type phenotype. Various countries have established an authority to monitor the operations connected with certification of plant propagative materials. Foundation Plant Services (FPS) in the United States of America and the Interprofessional Technical Center for Fruits and Vegetables (CTIFL) in France have

been entrusted with the responsibility of overseeing various operations carried out by nurseries and licensed propagators. The French National Certification Scheme of Citrus has been functioning since 1977 (Verniere 2000).

All plants for plantings in the case of deciduous fruit trees are produced by vegetative propagation. Once diseased plants are established in commercial orchards, the most effective control option is the removal of infected plants. Hence, use of disease-free seeds and propagative planting materials is the next effective disease management strategy in order to restrict disease incidence and spread. Certification programs are in operation in several countries for the production of disease-free nuclear stocks. Establishment of disease diagnostic centers (DDCs) is the basic requirement of the certification programs. Though conventional methods may be useful, adoption of modern molecular techniques is considered to be responsible for the dramatic enhancement in the levels of sensitivity, reliability and rapidity of disease diagnosis, increasing the credibility of the agency offering diagnostic service. For example, a multiplex PCR protocol using primers based on the sequences of *hrpF* gene could efficiently detect pathovars of *Xanthomonas campestris* involved in black rot disease of crucifers. This technique detected one infected seed present in seed lots of 10,000 healthy seeds (Berg et al. 2005). By applying a real-time PCR assay using specific primers based on the 16S–23S rDNA ITS sequences of different isolates, *Burkholderia glumae* was detected in rice seed lots and whole plants rapidly (Sayler et al. 2006). Another distinct advantage of employing molecular diagnostic methods is that they are amenable for automation facilitating testing of large number of samples and provision of conclusive results much earlier compared with the time required for traditional techniques. Furthermore, diagnostic kits have been commercially produced enabling the growers to use the tests right in their fields to determine the health status of their crops/planting materials.

There is practically no possibility of eliminating viruses/viroids from seeds/planting materials by applying chemicals. The feasible approach to prevent or reduce the disease incidence would be the use of seeds and planting materials that have been certified free of these pathogens. This approach has practical utility for horticultural crops that are propagated by stem cuttings, grafting or budding. The mother plants have to be indexed for the presence of all viruses infecting the particular crop. Stone fruit trees are affected by a large number of viruses belonging to different genera such as *Ilarvirus*, *Nepovirus*, *Trichovirus*, *Tombusvirus* and *Potyvirus*. In addition, two viroids *Hop stunt viroid* (HSVd) and *Peach latent mosaic viroid* (PLMVd) have also been reported to infect stone fruit trees. Both the viruses and viroids can be transmitted through planting materials. Diganostic methods for plant viruses based on nucleic acid sequences are being continuously improved. The stone fruit certification programs appear to be a compromise between simplicity of automation and sensitivity. The certifiable material may be assayed by serological or nonradioactive molecular hybridization methods. More sensitive techniques, however, are expensive as in the case of real-time PCR or microarray technology. These methods may be applied to test the primary sources or pre-basic materials as well as for imported dormant budwood during postentry quarantine or sanitation purposes (Pallás et al. 2000).

The French National Certification Scheme of Citrus functions at the International Technical Center for Fruit and Vegetables (CTIFL) under the authority of the Ministry

of Agriculture. The certification guarantees to the producers that the plant supplied, is free from the known diseases and true-to-type. More than 500 different varieties of citrus were regenerated by nucellar selection and later by shoot tip grafting and indexed for freedom from diseases using different techniques (Table 2.1). The variety is registered by allotting SRA number. The varieties which are conserved in outside plots or in an insect-proof structure are retested for tristeza and viroids. The Plant Protection Service (SPV) in charge of the phytosanitary passport, assists CTIFL in the sanitary controls in the nurseries (Verniere 2000). Bioassays on indicator plants have also been shown to be effective.

The Italian government, in 1991, promulgated a law concerning the constitutive regulation of the voluntary certification service of clonal propagation material for stone fruits, strawberry, olive, citrus, pome fruits and walnut. The harmonization of the directives of European Union was considered necessary. Particular attention has to be given to the identification of the most dangerous pathogens to be excluded from certified material, by providing guidance and technical advice on the most reliable methods for detection. The systemic pathogens such as viruses, phytoplasmas and viroids have to be carefully excluded or eliminated from the propagative materials, since they cannot be detected by visual observation and in some cases infection remain latent without any visible symptoms. Depending on the operative steps, rigorous nature of testing may vary. At the Center for the Conservation and Pre-multiplication (CCP), every plant of nucellar stock should have been found to be free of all pathogens listed. Each single plant is indexed and individual clone is identified by a number that is maintained during all propagation, production and commercial processing. The pre-base plants are transferred to pre-multiplication center (CP) for production of base plants. Multiplication center (CM) is responsible for producing certified mother plants from base plant material. Every year at least 10% of these plants are tested for virus and virus-like diseases. In the nurseries, the certified plant material is used for production of certified plants.

Effective certification program has to be based on reliable diagnostic methods. These methods may vary from conventional simple bioassays on herbaceous and woody plants to modern serological and nucleic acid-based techniques. Serological

Table 2.1 Techniques applied for the detection of citrus diseases for the certification plant materials in France

Diseases	Techniques
Tristeza virus	ELISA-polyclonal and monoclonal antibodies Dot-blot immunoassay
Exocortis and cachexiaviroids	Amplification on Etrog citron 861S1 + SDS-PAGE
Psorosis, Ringspot	Pineapple or Hamilan Sweet orange seedlings
Tatter leaf	Citrange Rustk or Citrus excelsa
Stubborn	ELISA, PCR

Source: Verniere (2000)

tests remain the simplest approach for an effective and rapid diagnosis. Among the immunoassays, ELISA format is the most frequently used technique for detection of microbial pathogens in plant materials. In the first two steps – CCP pre-base and CP base plants – the sanitary status of plants must be ascertained not only by ELISA, but also by biological tests on woody indicators. This is necessary because, even if one infected plant is missed, the fault could be enormously amplified, leading to generation of thousands of infected plants in the subsequent steps. In propagation center or in a nursery, serological tests are considered sufficient to assure the sanitary status of material under multiplication. Serology supports the workability of a certification scheme by allowing it to run a more reliable and fast diagnosis of the most important pathogens (Barba 1995). ELISA test was shown to be a technique of choice for certification or field surveys to determine the extent of infection by *Prune dwarf virus* (PDV). The efficiency of DAS-ELISA was equal to that of commercial kit (Abou-Jawdah et al. 2004).

In the United States, the California Department of Food and Agriculture (CDFA) has been authorized to administer the statewide California Registration and Certification (R & C) Program for *Vitis* and *Prunus*. The participants of the program, Foundation Plant Services (FPS), nurseries and licensed propagators have to follow all regulations of CDFA R&C Programs, in order to maintain planting materials in the program as foundation registered stock or as commercial registered increase blocks. The biological assays involving inoculation of herbaceous hosts and woody indicator plants are applied for the detection of plant viruses infecting grapevine and stone fruits. Among the molecular techniques, immunoassays and nucleic acid-based techniques have been employed. Of the two ELISA formats, the indirect method (DASI-ELISA) is favored due to its greater sensitivity, broader reactivity and convenience over DAS-ELISA format. The immunocapture (IC)-RT-PCR assay is the preferred technique for the detection of viruses. For the detection of *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd), the nucleic acid hybridization method using a complementary RNA (cRNA) as probe which forms duplexes of greater stability with target viroid RNA than DNA probes to the same target, is applied for testing the planting materials and mother plants (Rowhani et al. 2005).

The inspection time for seed potatoes for *Potato leaf roll virus* (PLRV) infection required was 5 weeks, when conventional tests were used. In contrast, the inspection time could be reduced to just one day by employing IC-RT-PCR protocol by capturing PLRV virions in the one-tube RT-PCR format. The *Thermus thermophilus* (*Tth*) DNA polymerase was used, instead of *Taq* DNA polymerase in this technique (Leone and Schoem 1997). The seed potato industry is faced with the problem of the emergence of new strains of *Potato virus Y* (PVY) resulting in possible rejection of seed tuber lots submitted for certification. The available RT-PCR formats were able to detect only certain combination of mixed strains infections in tubers. Hence, the need for the development of a single multiplex RT-PCR assay for detecting mixed infections by major strain types was recognized. One hundred and nineteen archived PVY isolates that had been characterized by serology and bioassay and/or previously published RT-PCR assays, were tested by this newly developed protocol. It was possible to resolve by using this new technique, the components of 16 mixed infections that

could not be detected by techniques developed earlier, indicating the suitability of this method for application in seed potato industry widely (Lorenzen et al. 2006).

In most countries, the certification of seeds and planting materials seems to be voluntary. However, national or regional (provincial) programs operate with a view to safeguarding the interest of the two stakeholders, viz., the producer who sells and the grower (farmer) who buys by ensuring the required levels of sanitary status in addition to maintaining the 'true-to-type varieties'. The voluntary certification programs in combination with adoption of strict quarantine regulations can be expected to result in high quality nursery stock with minimum regulatory infrastructure as in the United States of America. It would be more desirable, if the regional Plant Protection Organizations and the Food and Agriculture Organization (FAO) of the United Nations, make coordinated efforts to harmonize standards of international movement of plants and plant materials. Development of such national and international system may ensure the required protection for the growers and industry against all types of diseases (quarantined and non-quarantined) that can be transmitted through planting materials.

Appendix: Improved Direct Tissue Blot Immunoassay (DTBIA) for Rapid Detection of *Citrus tristeza virus* (CTV) (Lin et al. 2006)

i. Make sharp cut across the fresh young shoots of plants [healthy and infected by (CTV)]; press the freshly cut edge gently and evenly onto a nirtocellulose membrane for about 5 s, so that the impressions of the vascular bundles remain on the membrane and air dry the blots for 5 min.
ii. Incubate the blots with the prereaction solutions of CTV-specific antibodies [1/1,000 dilution for goat anti-mouse IgG-alkaline phosphatase conjugate (GAM-AP), 1/5,000 dilution for goat anti-rabbit IgG alkaline phosphatase conjugate (GAR-AP)] for 20 min and rinse with PBST buffer (0.15M NaCl, 0.015M sodium phosphate, pH 7.0, 0.05% Tween 20) for 5 min.
iii. Immerse the blots in solution containing nitroblue tetrazolium (14 mg) (NBT-BCIP) in substrate buffer (containing 0.1M NaCl and 5mM $MgCl_2$, pH 9.5) for 15–20 min.
iv. Stop the reaction by washing the blots in distilled water for a few seconds and observe under a light microscope at $\times 10 - \times 25$ magnification.
v. Observe development of purple color in the region of the blots associated with phloem cells indicating positive reaction and absence of purple color in the whole blot for negative reaction.

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Chapter 3
Genetic Resistance of Crops to Diseases

Abstract Management of crop diseases through disease resistance is acknowledged to be the most desirable and profitable approach. In order to develop cultivars with built-in resistance, the knowledge about the methods of assessing the levels of resistance to diseases caused by microbial pathogens and identifying resistance sources/genes is essentially required. The genetic basis of resistance to various diseases affecting crop(s) of interest, availability of resistance in cultivars or wild hosts, natural variations in pathogen populations, nature of plant-pathogen interaction and markers for identifying genotypes/lines possessing resistance genes can be studied by applying a range of molecular techniques. Use of molecular markers has been found to be very useful for selecting resistant plants rapidly even in seedling stage without waiting for the plants to reach the particular growth stage. In addition, the presence and contents of the pathogen DNA can be determined, using appropriate molecular technique well before the appearance of visible symptoms. The molecular bases of race-specific resistance, characterized by programmed cell death (PCD) and general resistance characterized by inducible defense responses exhibited in tissues far away from the infection site have to be clearly understood. The molecular methods have been successfully applied to understand various phenomena such as virulence and avirulence functions, role of elicitors, signaling systems induced in compatible and incompatible interactions, the posttranscriptional gene silencing and virus-induced gene silencing mechanisms, operating in plants and induction of various defense-related compounds and their action on microbial plant pathogens. The fungal, bacterial and viral pathogens have their own unique strategies to enhance their virulence and to overcome the host strategies to hamper their successful colonization of and establishment in plant tissues.

Among the various strategies of management of crop diseases, disease resistance is of immense practical importance. Use of cultivars with acceptable level of resistance to major diseases is considered as the best approach, since it can reduce or eliminate the expense and the effects of other chemical, physical, biological, cultural and regulatory control methods. Plants, unlike insect pests, are unable relocate, when conditions favourable for pathogen attack exist. The microbial plant pathogens have evolved different strategies to optimize plant metabolism to their advantage. Viruses

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exploit inter- and intra-cellular connection systems of plants to facilitate their systemic spread, whereas fungi interact with plant cells from extracellular site by forming infection structures such as appressoria, penetration or infection hyphae. On the other hand, bacteria have evolved a specialized type III secretion system (TTSS) which mediates the translocation of effector proteins into host cells. The effector proteins may be virulence determinants. Some of the Oomycetes fungi (*Phytophthora* spp.) have been reported to produce effector proteins with dual functions in pathogenicity or inducing resistance (Volume 2, Chapter 2). Plants have developed complex mechanisms to block the development of pathogen invasion effectively. The term 'resistance' is used to indicate the capacity of a plant species or cultivar to lessen the activity or harmful effects of a microbial pathogen. Plant disease resistance has been studied from different angles to integrate evolutionary, genetic, epidemiologic and economic conceptual frame work. Although a plant is exposed to numerous microbial pathogens, only a small proportion of them is able to induce disease successfully. The low frequency of disease indicates the existence of effective defense systems to contain the potential pathogens, suggesting that resistance may be considered the norm, while disease induction is the exception.

The first line of defense is seen at the whole plant level where the cells contain thick cuticle, thickened cell walls and other physical structures that obstruct pathogen development. The second line of defense may be recognized at the cellular level where a well coordinated, controlled and dynamic regulation of gene activity results in the synthesis of various proteins and antimicrobial compounds that restrict infection. Both preformed (passive) and active nature of defense processes are involved in the prevention of pathogen entering the plant or obtaining nutrition for its growth and reproduction. Several terms to differentiate two major forms of disease resistance such as constitutive and inducible, qualitative and quantitative, race-specific and nonspecific and inoculum-reducing and rate-limiting resistance have been used in literature, to describe similar phenomena using overlapping and sometimes confusing terms.

Resistance to diseases caused by microbial pathogens may be due to mechanisms controlled by '*R*' genes at one or a few loci designated monogenic, oligogenic or (polygenic) major gene resistance. Further, resistance may be expressed at different stages of plant/organ development. Adult plant resistance develops, when the plant reaches certain growth stage in its development. In general young plants (seedlings) are highly susceptible to viruses, but level of resistance to the viruses increases as the plants become older. So also the vegetable seedlings are susceptible to damping-off disease caused by *Pythium* spp. and this disease does not occur in the main field after transplantation. In contrast, unripe fruits are transiently resistant to fungal pathogens causing anthracnose and fruit rots. But as the fruits ripen, the pathogens find favourable conditions for their development and disease symptoms appear later (Narayanasamy 2002, 2006). Crop varieties differ in their levels of resistance to diseases caused by microbial pathogens. Likewise, pathogens exist in the form of several *formae speciales,* varieties, strains, races and biotypes. The applicability of molecular methods to differentiate pathogen population has been discussed in Volume 2, Chapter 3. The usefulness of molecular techniques in understanding the

genetic and the molecular basis of disease resistance observed in different crop plants will be discussed in this chapter.

The responses of host and nonhost plants to microbial pathogens have been studied in certain pathosystems. The development of a pathogen may be hampered by different mechanisms operating at different stages after a contact between the pathogen and plant has been initiated. Pathogen activities focus on colonization of the host and utilization of its resources, whereas plants are adapted to detect the presence of the pathogen and to activate its defense mechanisms in an orderly fashion to arrest the pathogen invasion. In some plant species or genotypes, the defense mechanisms operate so early and so thoroughly that the plant does not show any visible symptom of infection. Such plants are considered to be immune to the pathogenic species or strain. In some plant-pathogen combination, initiation of resistance mechanisms is delayed by different time interval resulting in corresponding low or higher degree of disease intensity designated high, moderate or low on a defined numerical scale. The plant species or genotype that allows unhindered development of the pathogen leading to production of characteristic disease symptom and pathogen proliferation is termed as susceptible.

3.1 Fungal Diseases

3.1.1 Genetic Basis of Resistance

3.1.1.1 Screening for Resistance

Crop disease management through development of cultivars with built-in resistance by incorporating appropriate genes is the cheap and environmentally safe approach. Farmers can grow such cultivars without any need for additional cost or expertise, since use of resistant varieties is compatible with cultivation practices and other methods of crop disease management. The basic requirement for the development of disease resistant cultivar is the availability of dependable source(s) of resistance. Identification of the genotypes possessing resistance genes can be achieved by using visual assessment and categorizing the entries into different groups based on the levels of resistance. Though the visual assessment is simple and easy to perform, it lacks accuracy and specificity. Further, it is not suitable for assessing resistance to individual pathogens in a disease complex. Dependence on visual assessment may lead to misleading conclusion on the resistance of genotypes when symptoms are nonspecific and likely to be confused with stress-related symptoms as in the case of wheat leaf blotch disease complex caused by *Septoria tritici* and *Stagonospora nodorum*. The coalescing lesions and pale pycnidia associated with infection by *S. nodorum* may be difficult to differentiate from natural senescence. Immunoassays and nucleic acid-based techniques, on the other hand, do not rely on the development of visible symptoms (Narayanasamy 2001).

The pathogens biomass present in plants can be quantified and related to the level of resistance of the test plant. There is a negative relationship between fungal biomass and resistance to the pathogen. Enzyme-linked immunosorbent assay

(ELISA) has been applied to quantify fungal antigen in resistant and susceptible cultivars and genotypes, after inoculation with the pathogen. A polyclonal antiserum was developed using mycelia and zoospores of *Aphanomyces euteichus* infecting peas. The antiserum specifically reacted with *A. euteichus*, but not with *Phytophthora, Fusarium* and *Pythium.* The relationship between the rate of lesion development and pathogen population was determined by ELISA tests. The lesion length on the main roots of peas infected by *A. euteichus* was positively correlated with antigen concentration ($R^2 = 0.91$) as determined by ELISA test at A405 nm. Resistance to root rot disease appears to be related to the reduction in oospore production, pathogen multiplication and zoospore germination and slower lesion development (Kraft and Boge 1994, 1996).

Anisogramma anomala causing eastern filbert blight disease in hazelnut (*Corylus avellana*) requires a long incubation period of 13–22 months for symptom expression, making it difficult for assessment of resistance of germplasm types. An indirect ELISA protocol was developed for the detection of *A. anomala* in inoculated plants before the appearance of visible symptoms. It was demonstrated that the pathogen could be detected by ELISA format, at 3–5 months after inoculation when no symptom of infection could be observed, thus reducing the period of observation very significantly. By applying the ELISA test in a breeding program, a hazelnut progeny with a gene conferring high level of resistance derived from the cultivar, Gasaway was identified (Coyne et al. 1996). The levels of resistance of test entries of narrow-leaved lupins (*Lupinus angustifolius*) to latent stem infection by *Diaporthe toxica* were assessed using a competitive ELISA procedure. Inoculated stem pieces incubated in moist chamber for 6 d were tested by ELISA format. The results of ELISA tests were highly correlated ($r = 0.92$) to those obtained by microscopic examination after a 21-day incubation period. The test entries were classified as susceptible, resistant and highly resistant based on the antigen concentration (Shankar et al. 1998).

The effectiveness of ELISA technique for determining the resistance of sugarcane cultivars to the red rot disease caused by *Colletotrichum falcatum* was demonstrated. This immunoassay provided reliable results much earlier than the conventional method (Viswanathan et al. 2000). The possibility of using ELISA to quantify the populations of *Cladosporium fulvum* in tomato leaves (Kaprovich-Tate et al. 1998), *Magnaporthe grisea* in rice leaves (Hasegawa et al. 1999), *Botrytis cinerea* in pear stems (Meyer et al. 2000) and *Peronospora parasitica* in tobacco leaves (Carzaniga et al. 2001) has been indicated, suggesting their possible application for assessment of resistance of cultivars and germplasm entries.

A specific and quantitative biotin/avidin (BA)-enzyme linked immunosorbent assay (ELISA) was developed to assess the levels of resistance of wheat and triticale cultivars to *Septoria tritici* (causing leaf blotch) and *Stagonospora nodorum* (causing leaf and glume blotch) under field conditions. The antigen contents of *S. tritici* and *Stagonospora nodorum* were determined in the flag leaf (F) and the first leaf below the flag leaf (F-1) at a predetermined growth stage on five triticale and 11 wheat cultivars. *S. nodorum* was predominant in triticale cultivars, whereas both pathogens were commonly detected in wheat cultivars by BA-ELISA tests. The BA-ELISA

values correlated well with the susceptibility ratings in the cultivar list recommended by the German Federal Office of Plant variety (Bundessortenant), based on visual assessment. The BA-ELISA test can be used before visible symptoms appear and also when disease pressure is low. Furthermore, this technique can distinguish these two fungal pathogens and provide accurate assessment of intensities of symptoms induced by them individually. As the BA-ELISA gives useful information on the quantitative differences between cultivars, it will be possible to grade the levels of resistance of cultivars and genotypes (Tian et al. 2005b). The BA-ELISA format has the potential for use in assessment of levels of resistance of crop cultivars and genotypes in breeding programs.

The nucleic acid-based techniques have been shown to be more sensitive, specific, rapid and reliable compared to immunoassays for detection, differentiation and quantification of microbial pathogens. Real-time PCR, directly estimates fungal DNA from total DNA extracted from infected plant tissues. Hence, this technique can be applied to measure fungal development even before any visible symptoms appear. The alfalfa plants inoculated with *Phytophthora medicaginis* were classified as either resistant or susceptible based on visual assessment of disease response. A real-time fluorescent PCR assay employing a set of specific primers and fluorochorme-labeled probe (TaqMan) was developed to quantify the fungal pathogen in the infected alfalfa plants. The assay provided estimates of fungal DNA in alfalfa populations with different levels of resistance based on the analysis of DNA extracted from the roots of bulked plant sample. The pathogen DNA content was significantly less in highly resistant plants than in susceptible plants. The procedure developed in this study can be useful for breeding programs as well as for studying microbial population dynamics in plants simultaneously infected with different pathogens (Vandemark and Barker 2003). A real-time polymerase chain reaction (PCR) procedure was developed for the detection and quantification of *Phytophthora capsici* causing *Phytophthora* root rot of pepper (chilli) plants. By using the real-time PCR assay with SYBR Green Supermix (BioRad) and specific primers for *P. capsici,* the pathogen DNA could be detected as early as 8 h post-inoculation. The increase in pathogen DNA was more rapid in susceptible cultivars and slower in resistant genotypes (PI1201234 and SCM331). The amount of pathogen DNA quantified in each pepper genotype was correlated with the levels of susceptibility (positive)/resistance (negative). The relationship between pathogen virulence and extent of plant tissue colonization was also discernible. The results clearly showed that the real-time PCR with SYBR Green was sensitive and robust enough to determine both pathogen development and to assess the levels of resistance of pepper cultivars and genotypes to *P. capsici*. Consequently the most resistant genotype(s) may be selected and used as sources of disease resistance in breeding programs (Silvar et al. 2005).

A pathogenicity assay based on the relative quantification of fungal and plant DNA in infected *Arabidopsis thaliana* leaves was developed based on real-time quantitative PCR. The progression of *Alternaria brassicola* and *Botrytis cinerea* (to which *A. thaliana* is resistant and susceptible respectively) was determined by performing two real-time PCR reactions targeted at fungal and plant sequences on

inoculated samples. In the case of *A. brassicola* no significant variation in fungal DNA contents could be detected over the time course of infection. In contrast, during the course of infection, *B. cinerea* DNA became predominant in the infected samples and this was ascribed to both fungal proliferation and massive plant cell collapse. The fungal abundance could be reliably quantified from the very early stage of infection. The DNA extraction method followed was less expensive, but yielded quality DNA suitable for the assay. The protocol developed in this investigation was highly sensitive and reliable and exhibited high robustness. This technique may be useful to discriminate between lines displaying slightly different levels of resistance (Gachon and Saindrenan 2004).

Various investigations have revealed variations in resistance of crop plant species/varieties and aggressiveness (virulence) of pathogen species/strains/races/biotypes. In order to understand more clearly the interaction between host plant and pathogen, the precise identity of the pathogen isolates has to be established, in addition to host plant identity. *Colletotrichum* crown rot disease of strawberry may be caused by *Colletotrichum gloeosporioides, C. fragariae* or *C. acutatum.* The resistance of strawberry cultivars to *C. gloeosporioides* isolates was investigated by identifying the pathogen isolates using a species-specific internal transcribed spacer (ITS) region primer and RAPD analysis. The bands from five RAPD primer amplification reactions were scored for each isolate. Two distinct clusters of isolates of *C. gloeosporioides* and *C. fragariae* could be recognized based on RAPD banding pattern distinguishable from one another, indicating that they were not asexually produced from the same parent strain. Pathogenicity tests over a period of 3 years using 12 distinct *C. gloeosporioides* isolates and 10 strawberry cultivars showed differences in disease resistance among cultivars and differences in aggressiveness among pathogen isolates. The cultivar 'Treasure' exhibited high level of resistance to both *C. gloeosporioides* and *C. fragariae* (Mackenzie et al. 2006).

Phoma medicaginis infects *Medicago sativa* (alfalfa) and *M. truncatula*, a legume grown widely in Australia, causing black stem and leaf spot diseases. Five gene regions of *P. medicaginis*, actin, β – tubulin, calmodulin, translation elongation factor-1α (EF-1α) and internal transcribed spacer rDNA were examined to compare the sequences of eight isolates. Sequence comparisons indicated that the isolates formed a distinct group. EF-1α contained a hypervariable 55-bp repeat unit which could be used as a basis for differentiating the isolates using a rapid PCR-based protocol. The nested primers designed to EF-1α amplified within an intron where duplication of noncoding DNA was responsible for the hypervariable nature of this gene region. Three genotype groups of isolates based on the variation in the sequence of EF-1α were recognized. Three isolates selected as representatives of each of the three genotype groups were inoculated on 86 accessions of *M. truncatula* to assess their levels of resistance/susceptibility to *P. medicaginis*. Two accessions Sa 1489 and Sa 8623 showing resistance to the three representive isolates, were selected as dependable sources of resistance to *P. medicaginis* (Ellwood et al. 2006).

The light leaf spot disease in winter oilseed rape is due to *Pyrenopeziza brassicae* (anamorph – *Cylindrosporium concentricum*) and it is responsible for serious losses in UK, Germany and other European countries. Visual assessment of *P. brassicae*

infection is not possible before necrotic lesions appear. Hence, plants are incubated at high humidity for several days (Fitt et al. 1998). In addition, resistance of cultivars determined based on inoculation of seedling cotyledons does not have any relation with resistance exhibited by adult plants (Bradburne et al. 1999). Hence, a method of assessing resistance to light leaf spot by detecting symptomless infection by PCR technique can be effective as in the case of *Leptosphaeria maculans* (Kenyon et al. 2004). A new, more sensitive primer pair PbITSF/PbITSR was found to be effective for the detection of *P. brassicae* infection in symptomless oilseed rape tissue. This protocol could detect the pathogen both in aritifically inoculated and field samples rapidly and reliably. The field resistance of oil seed rape cultivars and genotype can be assessed and cultivars with good field resistance can be introduced in countries where the light leaf spot is a serious problem limiting production of oilseed rape (Karolewski et al. 2006).

Oat crops are severely affected by the crown rust disease caused by *Puccinia coronata* f.sp. *avenae*. A new quantitative method was considered necessary, since the results obtained through visual assessment required experience, in addition to being time-consuming and laborious. The new method involves simple inoculum application, quantitative sampling from inoculated areas, a closed tube DNA extraction method restricting loss of tissue and real-time PCR using a pathogen specific TaqMan primers/probe set which amplified a 75-bp fragment only from *P. coronata* isolates. No amplification was visualized in reactions containing DNA templates from *P. graminis* f.sp. *avenae* and *P. triticina,* indicating the specificity of the TaqMan assay (Fig. 3.1). The results of fungal DNA (FDNA) estimations by the real-time PCR and image analysis of genotypes with different resistance levels were compared. The susceptible, moderately resistant and resistant genotypes could be distinguished at seedling and/or adult plant stages using FDNA estimations. In contrast, digital image analysis did not provide such a separation of genotypes. Furthermore, the fungal development could be detected earlier and more rapidly using FDNA assessment in genotypes with lower levels of resistance. Fungal development in the susceptible genotypes was at a faster rate than in resistant genotypes as

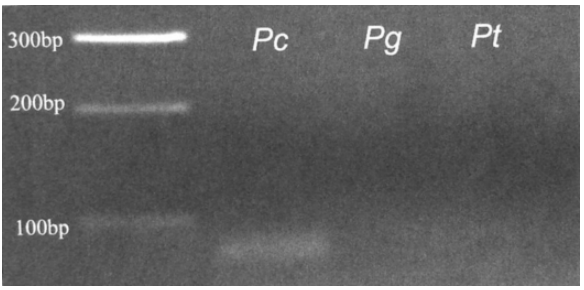

Fig. 3.1 Assessment of primer specificity using real-time PCR product visualization from reactions containing *Puccinia* (Pc), *P. graminis* f.sp. *avenae* (Pg) or *P. triticina* (Pt) DNA
Molecular size markers are at the left extreme. (Courtesy of Jackson et al. 2006; The American Phytopathological Society, St. Paul, MN, USA)

revealed by real-time PCR assay. The resistance levels could be graded even from four days after inoculation and this is not possible by applying conventional assessment methods. This assay protocol may be a valuable research tool for studying other aspects of host-pathogen interaction (Jackson et al. 2006).

Soil-borne fungal pathogens such as *Rhizoctonia solani*, *Fusarium solani* and *Aphanomyces euteichus* cause root rot diseases in many food legumes, pea, chickpea, lentil, fababean and lupin limiting their yield in many countries world wide. The use of resistant cultivars is the best option for the management of these diseases, since it is the safest and most economical strategy. Effective and precise screening procedure has to be adopted for assessing the levels of the resistance of available germplasm collection including wild species. In addition, the knowledge of both host plant and pathogen biology, variability, host-pathogen interaction, genetic structure and geographic distribution is also essential. The conventional method of raising the test entries in sick plots (SPs) and visual scoring using the percentage of plants affected as the basis is considered to be more practicable. Molecular methods have limited application as a selection tool for resistance, especially if mass scale germplasm evaluation has to be taken up in the field. However, the possibility of employing molecular techniques for assessing resistance of genotypes has been demonstrated for some pathosystems. The potato cultivars showing resistance to late blight pathogen *Phytophthora infestans* could be differentiated by measuring β-glucuronidase (GUS) activity (De la Peña and Murray 1994). Likewise, variations in resistance levels of wheat cultivars to *Pseudocercosporella herpotrichoides* were determined based on GUS activity (Kamoun et al. 1998a). In a later study, the usefulness of GUS activity for assessing the levels of resistance of tomato lines to *Fusarium oxysproum* f.sp. *radicis-lycopersici* (FORL) causing wilt disease was examined. The β-glucuronidase (*gus*) reporter gene was integrated into FORL in a co-transformation experiment using hygromycin B resistance (*hph*) gene as selective marker. Of the ten stable transformants, F30 showed a strong positive correlation between GUS activity and accumulation of biomass in in vitro grown FORL. A parallel increase in lesion development and GUS activity was observed in a susceptible and resistant tomato line. However, the levels of GUS activity in resistant line were greater than in susceptible line, indicating that GUS activity cannot be used as a basis of differentiating the resistance of tomato lines to the wilt pathogen (Papadopoulou et al. 2005).

Real-time PCR assay has been applied to quantify fungal pathogens in plant tissues as well as in soils. The method allowed detection and quantification of *Aphanomyces euteiches* in pea and a significant correlation between the amount of pathogen DNA and disease severity index for three isolates was established. However, no such correlation was evident for the two other isolates tested (Vandemark and Grünwald 2005). In the soybean-*Fusarium solani* f.sp. *glycines* pathosystem, the application of qualitative PCR format was found to be useful in grading resistance to the pathogen (Gao et al. 2004). Characterization of isolates of *Fusarium oxysporum* f.sp. *ciceris* (*FOC*) belonging to two pathotypes causing different yellowing and wilting symptoms was possible with the use of the random amplified polymorphic DNA (RAPD) technique. The isolates could be separated into two

clusters corresponding to the two biotypes. Race-specific RAPD bands were identified and used for the design of sequence characterized amplified region (SCAR) primers specific for races 0, 1B/C, 5 and 6 (Jiménez-Gasco et al. 2001; Jiménez-Gasco and Jiménez-Diaz 2003).

Since the responses of cultivars and genotypes vary significantly depending on the race, strains or biotypes of the pathogen, precise identification of the isolates is necessary to determine the levels of resistance of the host plants. The molecular identity of *P. infestans* strains infecting potatoes in the Toluca Valley of Mexico was examined. The isolates of *P. infestans* obtained from five potato varieties with differing levels of disease resistance were compared based on mating type (MT), isozyme genotype for glucose-6-phosphatase isomerase (Gpi) and peptidase (Pep) and sensitivity to metalaxyl. In addition, host resistance components and specificity on the host-pathogen interaction were assessed using detached leaves in the laboratory and attached leaves in greenhouse for three of the potato varieties. Every genetically derived host factors such as efficiency of infection, number and size of lesions, latent period or time required for sporulation after infection and sporulation capacity that adversely affected the severity of disease were considered as host resistance component. These variables were found to be useful to measure the pathogenic fitness of the pathogen isolates as well as the level of host resistance. The isolate from cv. Alpha (susceptible) did not infect cv. Norteña (resistant) in the laboratory and greenhouse trials. This indicated a gene-for-gene interaction, where cv. Norteña has a major or vertical resistance genes for corresponding virulence genes of the pathogen. The cv. Alpha does not have *R* genes and hence could be infected by wild type, virulent and avirulent pathogen genotypes alike. No relationship between a specific mating type and host resistance was evident. The field resistance exhibited by cvs Rosita and Norteña was lost as revealed by greenhouse tests under artificial inoculation conditions. The leaf tissue of both varieties were equally damaged as that of susceptible variety. However, inhibition of sporulation was the only host resistance component identified in the greenhouse for the varieties with field resistance. The cvs. Rosita and Norteña have consistently remained resistant under natural conditions of Toluca valley (Grünwald et al. 2002), questioning the validity of laboratory and greenhouse tests for screening potato test entries for selection of genotypes with field resistance (Lozoya-Saldaña et al. 2006).

The gene-for-gene interaction between host plant and microbial pathogens has been demonstrated in over 20 pathosystems and possibly, such interaction functions in many more, since the classical genetical study of Flor (1956) on flax-rust pathosystem. Resistance may be determined by individual members of families of dominant genes, each member conferring resistance to specific strains of the pathogen. A basic defense strategy in plants is the ability to perceive a diverse range of elicitor molecules (Dangl and Jones 2001). There is a robust correlation between elicitor perception and resistance to pathogens in gene-for-gene interactions and such recognition occurs only in resistant host genotypes. This type of elicitors are designated specific elicitors and are frequently the direct products of corresponding avirulence genes of the pathogen (Bonas and Lahaye 2002). In the case of general elicitors, the presence of pathogen is perceived by both susceptible and resistant

plants and they may contribute quantitatively to defense response of the host plant. One of the components of resistance of *Nicotiana* spp. is the recognition of elicitin of pathogens origin (*Phytophthora* spp). The involvement of elicitin in the development of resistance to *P. infestans* in potato and tomato and to *P. parasitica* var. *nicotianae* in tobacco has been demonstrated (Ricci et al. 1992; Kamoun et al. 1993).

Screening *Solanum* germplasm for response to elicitins from *P. infestans* (INF elicitins) was taken up to facilitate the genetic dissection of chitin response in plants. For this purpose, the use of the *Agrobacterium tumefaciens* binary PVX expression system (PVX agroinfection) was adapted and optimized. This assay was applied to identify a total of 11 clones of *S. huancabambense* and *S. microdontum* that responded to the elicitors INF1, INF2A and INF2B of *P. infestans.* As in the case of *Nicotiana* spp. tested earlier, *S. huancabambense* and *S. microdontum* clones also showed hypersensitivity-like cell death following infiltration with purified recombinant INF1, INF2A and INF2B, thereby validating the screening technique. The natural variation in response to INF elicitins in the identified *Solanum* accessions was exploited for evaluating the relationship between INF recognition and late blight resistance. Since several INF-responsive *Solanum* plants were susceptible to *P. infestans*, it appears that INF elicitins have to be considered as general elicitors and they do not have a measurable contribution to resistance to late blight disease in *Solanum* spp. (Vleeshouwers et al. 2006).

3.1.1.2 In Vitro Selection for Disease Resistance

Somaclones generated from individual cells or organ tissues show variations in several characteristics including resistance to pathogens or their metabolites. In order to identify differentially expressed sugarcane genes upon inoculation with sugarcane smut pathogen *Ustilago scitaminea,* cDNA–AFLP and suppression subtractive hybridization (SSH) techniques were employed. Markers were developed using an RFLP approach for characterizing sugarcane genotypes for use in breeding programs. Fifty nine polymorphisms related to smut resistance were identified (Butterfield et al. 2004). Sugarcane somaclonal variants displaying resistance to smut disease were examined to gain deeper insight into the genetic background of enhanced resistance. A study to identify genes that are activated and associated with resistance in sugarcane following inoculation with either *U. scitaminea* or *Bipolaris sacchari* (causing eye spot disease of sugarcane) was taken up. Differentially expressed transcription-derived fragments (TDFs) that were induced following challenge of resistant and susceptible somaclones were isolated using a cDNA-AFLP approach. Of the 62 differentially regulated genes, ten were down-regulated, while 52 were induced and these differentially induced TDFs were isolated and sequenced. Of these TDFs, 11 were isolated from the smut-resistant somaclone, whereas eight were isolated from eye-spot resistant somaclone (Borrás-Hidalgo et al. 2005).

The differential expression of TDFs (19) was confirmed by Northern blots prepared with total RNA that was also used for the cDNA-AFLP analysis. The mRNA

levels of genes that were homologous to seven of those TDFs were highly induced in resistant somaclones inoculated with *U. scitaminea* or *B. sacchari,* whereas only low or no expression was discernible in the susceptible parental lines as revaled by Northern blot analysis. Only one of the 52 differentially expressed TDFs, was highly induced in both *U. scitaminea* and *B. sacchari* interactions with sugarcane somaclones. The TDF was found to be derived from a gene encoding a putative serine-theronine kinase. The genes that might contribute to disease resistance, identified in this study have the potential for use in breeding programs (Borrás-Hidalgo et al. 2005).

The microbial plant pathogens produce nonspecific phytotoxins or host specific pathotoxins. As the toxin production by the fungal pathogen is essential to cause serious disease symptoms, it is considered that making the host resistant to the toxins would also make it resistant to the disease (Alexander et al. 1997). The resistance mechanisms to toxin-producing fungi may be based on the ability of the product of an *R* gene to inactivate a toxin that may generally induce necrosis or may inhibit the induction of active defense responses (Takken and Joosten 2000). The use of toxins for selecting resistant genotype of this host plant species in vitro depends on (i) production of the toxin(s) by all identified isolates of the fungal pathogens and (ii) induction of primary symptoms similar to that caused by the fungus. The principal advantages of using in vitro selection of disease resistant genotypes are (i) unfavourable environmental conditions can be avoided; ii) the concentration of toxins for the assay can be optimized; (iii) a large number of individuals may be tested in a small space; (iv) manipulation of mutants, haploids, somaclones with higher variability in the host genome is possible and (v) mass screening of mutants for resistance can be effectively performed. About 30 plant species including herbs and woody plants from various families have been screened in vitro with a wide range of selective agents such as toxins and elicitors for the selection of resistant genotypes that can be used as sources of resistance in breeding programs (Švábová and Lebeda 2005) and some of them are presented in Table 3.1.

Some of the fungal pathogens are known to produce host-specific toxins (HSTs) that can induce most of the symptoms caused by the pathogen in susceptible plants. Different *Helminthosporium* spp. such as *H. victoriae, H. maydis* race T and *H. sacchari* respectively produce HSTs, victorin, HMT-toxin and helminthosporoside which can induce characteristic symptoms in susceptible oat, maize (Texas male sterile lines cytoplasm) and sugarcane. Oat varieties developed with Victoria genes were highly susceptible to victorin, whereas cv. Bond resistant to *H. victoriae* was insensitive to victorin. Resistance to Victoria blight was governed by a single major gene, but resistance to toxin was considered to be specific, horizontal and monogenic in many cases (Vanderplank 1984). The resistant oat plants did not allow the build up of toxin level to reach the threshold value required for production of disease symptoms. Hence, cv. Bond was not attacked by the pathogen. Other oat varieties also showed similar response to the toxin victorin. *H. victoriae* is not known to produce many new races capable of infecting the resistant varieties. Hence, resistance to *H. victoriae* in oats is found to be durable and depends on the insensitivity to the toxin produced by the pathogen (Parlevliet 1993).

Table 3.1 In vitro selection of genotypes exhibiting resistance to fungal pathogens

Crop Plant	Pathogen (selective agents)	References
Alfalfa (*Medicago sativa)*	*Colletotrichum trifolii*	Cucuzza and Kao (1986)
	Verticillium albo-atrum	Ireland and Leath (1987)
Apple (*Malus domestica*)	*Phytophthora cactorum*	Mezzetti et al. (1992)
Banana (*Musa*)	*Mycosphaerella fijiensis*	Okole and Schulz (1997)
Barley (*Hordeum vulgare*)	*Drechslera teres*	Hunold et al. (1992)
Carnation (*Dianthus caryophyllus*)	*Fusarium oxysporum*	Thakur et al. (2002)
Chickpea (*Cicer arietinum*)	*Fusarium oxysporum f.sp ciceri*	Rao and Padmaja (2000)
Common bean (*Phaseolus vulgaris*)	*Colletotrichum lindemuthiamum*	Fernandez et al. (2000)
Cotton (*Gossypium*)	*Verticillium dahliae*	Zhen and Li (2004)
Egg plant (Brinjal) (*Solanum melongena*)	*Verticillium dahliae*	Koike et al. (1993)
Grapevine (*Vitis vinifera*)	*Elisinoe ampelina*	Jayasankar et al. (2000)
Mango (*Mangifera indica*)	*Colletotrichum gloeosporioides*	Jayasankar et al. (1999)
Oat (*Avena sativa*)	*Helminthosporium victoriae*	Rines and Luke (1985)
Pineapple (*Anans cosmosus*)	*Fusarium subglutinans*	Borrás et al. (2001)
Potato (*Solanum tuberosum*)	*Verticillium albo-atrum*	Koike et al. (1996)
Rape (oilseed) (*Brassica napus)*	*Phoma lingam*	Sjödin and Glimelius (1989)
Soybean (*Glycine max*)	*Septoria glycines*	Song et al. (1993, 1994)
Sugarcane (*Saccharum officinarum*)	*Colletotrichum falcatum*	Mohanraj et al. (2003)
Tobacco (*Nicotiana tabacum)*	*Phytophthora cryptogea*	Bonnet et al. (1985)
	Alternaria alternata	Ishida and Kumashiro (1988)
Tomato (*Lycopersicon esculentum*)	*Fusarium oxysporum*	Shahin and Spivey (1986)
Wheat (*Triticum aestivum*)	*Septoria nodorum*	Keller et al. (1994)

The toxins victorin and HMT-toxin produced by *H. victoriae* and *H. maydis* are present in the conidia and they are released at the time of conidial germination (Nishimura and Scheffer 1965; Comstock and Martinson 1975). In contrast, HC-toxin produced by *H. carbonum (Cochliobolus carbonunum)* race 1 causing maize leaf spot disease is not detectable in the conidia. But this toxin could be isolated in the leaf extract from susceptible maize plant. However, it was not possible to detect its presence in the leaf extract of resistant plant at any time after inoculation (Dunkle et al. 1991). By using Plasma Desorption Mass Spectrometry (PDMS), the release of HC-toxin at the time of formation of mature appressoria of *H. carbonum* was demonstrated (Weiergang et al. 1996). The *Hm1* gene codes for carbomyl reductase that inactivates HC-toxin (Johal and Briggs 1992; Meeley et al. 1992). In a later study, secretion of HC-toxin by *H. carbonum* during appressorium formation was

confirmed. The *Hm1* gene was constitutively expressed. Because of the presence of HC-toxin on the leaf surface of resistant plant in substantial quantity, the fungus failed to become established in the resistant plants even after 36 h. The results suggest that while *Hm1* gene is expressed constitutively at low levels, it is rapidly induced in response to infection resulting in nearly immediate cessation of pathogen growth (Weiergang et al. 2004).

Some fungal plant pathogens produce HSTs that have been shown to be primary determinants in pathogenesis and capable of inducing typical disease symptoms in the absence of the pathogen concerned. Culture filtrates (CFs) containing the toxins or purified toxins have been used for in vitro selection and regeneration of disease – resistant plants (Binarova et al. 1990; Vidhyasekaran et al. 1990). In the absence of any known source of resistance, the alternative approach of using CFs to screen tissue cultures (calluses) for resistance to the fungal pathogen appears to be feasible. Soybean plants resistant to *Septoria glycines* causing brown spot disease were obtained by using the host-specific toxin (HST) produced by the pathogen. The culture filtrates induced typical brown spot disease symptoms on soybean leaves and cotyledons and inhibited the growth of soybean calli (Song et al. 1993). Two sources of plant materials, immature embryo and mature seed were exposed to the pathotoxic CF. Soybean plants with resistance to *S. glycines* were regenerated by organogenesis from immature embryos of the cv. BSR201 and from mature seeds of three genotypes BSR201, Fayette and L1615. Field evaluation of R_2 and R_3 resistant plants did not exhibit symptom of infection until R_6 growth stage. The incubation period was at least 5 weeks longer than in comparable susceptible plants. Among the 3 cultivars tested, only progeny from BSR 201 inherited resistance to brown leaf spot disease (Song et al. 1994). The possibility of selecting cotton cultivars resistant to *Verticillium dahliae* using VD-toxin for treating the calluses was reported by Zhen and Li (2004).

3.1.1.3 Identification of Resistance Genes

Classical genetical studies have shown that resistance genes are clustered in the genomes of several species exhibiting resistance to pathogen(s). They may be either in groups of genetically separable loci or apparently multiallelic series (Islam and Shepherd 1991; Jørgensen 1992). Different genes within the same cluster may determine resistance to taxonomically diverse pathogens. Rapid developments in molecular genetics have facilitated the identification and mapping of resistance genes on plant chromosomes. Cloning disease resistance genes from several plant species has been achieved paving the way for studying the components of signal transduction pathways (Bent 1996; Jones and Jones 1997).

It has been comparatively easy to select for resistance mechanisms controlled by genes at one or a few loci – monogenic, oligogenic or major gene resistance encoded by *R* genes. The major draw-back of this type of resistance is that the pathogens causing diseases in such host plants are able to overcome this resistance by producing new physiologic races compatible with *R* genes. Selection for monogenic

resistance based on a different receptor encoded by a different *R* genes will lead to natural selection for mutants without the corresponding elicitor. This in turn may result in loss of resistance due to emergence of another new race of the pathogen. Such an evolutionary battle between wheat cultivars with monogenic resistance and stem rust pathogen is a classical example (Stakman and Harrar 1957). The presence of a set of dominant *R* genes in the host with a matching set of dominant *avr* genes in the pathogen has been reported in several pathosystems (Narayanasamy 2002). All known *R* genes (more than 40 genes) can be grouped into five classes based on their sequence structure and functional domain/motifs. Most of these *R* genes belong to the nucleotide-binding site NBS-leucine-rich repeat (LRR) type (Hammond-Kosack and Jones 1997; Martin 1999).

Many resistance genes contain similar sequence motifs, although they determine resistance to very different pathogens. Because of the presence of conserved domains in resistance genes, it has been possible to clone several additional resistance genes from diverse species by polymerase chain reaction (PCR) with degenerate oligonucleotide primers to the conserved motifs. The progenies of crosses may be prescreened by DNA testing to identity those carrying known resistance gene or chromosomal sequences closely linked to such a gene. This process is termed marker-assisted selection (MAS) (Henry 1997). This *R* gene class consists of cytoplasmic proteins with predicted leucine zipper, nucleotide-binding site (NBS) and C-terminal leucine-rich repeat motifs. This class includes *RPS2* and *RPM1* of *Arabidopsis* and *Fusarium oxysporum* resistance gene 12 of tomato (Bent et al. 1994; Bent 1996; Grant et al. 1995; Ori et al. 1997). The R genes *I*-1 and *I*-2 conferring resistance to races 1 and 2 respectively of *F. oxysporum* f.sp. *lycopersici* (FOL) were mapped to chromosome 11 in tomato. The *I*-2 gene cluster has one functional copy and six nonfunctional homologs of *I*-2 gene. Primers designed based on the sequences of the functional copy were employed in a multplex PCR assay to differentiate tomato genotypes with *I*-2 gene from the ones without this gene. Based on the presence or absence of this marker, 39 out of 40 tomato genotypes could be differentiated. The cv. Plum Crimson carrying the *I*-3 gene was the exception and it showed resistance to three races 1, 2 and 3 of FOL. Validation of the results obtained in this investigation in three countries and by bioassays adds merit to the effectiveness of the MAS approach in disease resistance programs (El Mohtar et al. 2007). Another *R* gene class encloses the receptor-like kinases (RLKs) that have an extracellular LRR and an intracellular serine-threonine kinase domain. The rice genes *Xa21* and *Xa26*, conferring resistance to bacterial leaf blight (BLB) disease (Sun et al. 2004) and the *Arabidopsis FLS2*, an RLK gene involved in the perception of the bacterial elicitor flagellin (Gómez-Gómez and Boller 2000) belong to this class of resistance genes.

The PCR assay using the degenerate primers has been applied to amplify *R* gene analogs (RGAs) from many plant species. This approach was used extensively to clone genomic copies after PCR amplification of NBS–LRR sequences and then to sequence and map via RFLP in different plant species (Kanazin et al. 1996; Leister et al. 1996, 1998; Rivkin et al. 1999; Deng et al. 2000). Most of the cloned RGAs in *Arabidopsis* were shown to be genetically linked with disease resistance loci (Aarts

et al. 1998). Likewise, two RGAs were mapped to known disease resistance clusters in lettuce (Shen et al. 1998). The NBS-LRR containing sequences were found to be clustered in many plant species and similarly clustering of functional *R* genes has also been reported. The *Cf* genes of tomato (Jones et al. 1994; Dixon et al. 1998), *Dm* genes of lettuce (Meyers et al. 1998) and resistance genes to different pathogens at three genomic regions in potato (Gebhardt and Valkonen 2001) were observed to be in clusters.

All-stage resistance to all races of barley stripe rust pathogen *Puccinia striiformis* f.sp. *hordei* (PSH) was located in the barley genotype BBA 2890. A single recessive gene *rps1.a* was shown to control this resistance in BBA 2890. By using chromosome-specific simple sequence repeat (SSR) markers, attempts were made to identify resistance gene analog polymorphism (RGAP) marker for all-stage resistance gene *rps1.a* and to map this gene on the chromosome of barley. Seedlings of the parents and 200 F_8 recombinant inbred lines (RILs) were tested for reaction to races PSH-14, PSH-48 and PSH-54 in the greenhouse. Twelve primer pairs generating repeatable polymorphic bands were applied for generating the 150 F_8 RILs. A genetic linkage group was constructed for the resistance gene with 13 RGAP markers and four chromosome-specific SSR markers. The closest RGAP marker for the resistant allele was within a genetic distance of 2.1 centimorgans (cM). It is possible, by using the genetic markers to incorporate the resistance gene into selected barley cultivars and also to enhance the level of resistance of barley lines to PSH by pyramiding this gene with other resistance genes (Yan and Chen 2007).

Molecular markers allow the dissection of monogenic and quantitative resistance. Molecular markers are useful for preserving and exploiting germplasm, marker-aided selection (MAS) of resistance genes and gene deployment for increasing levels of resistance of cultivars to desired pathogen(s). By adopting MAS, it will be possible to effectively deploy resistance genes to provide stable resistance to different economically important diseases affecting major crops. All regions of the plant genome can be assayed for linkage to resistance, using molecular markers. Resistance to diseases has been found to be linked with undesirable traits. Mapping of the genome using molecular markers helps to identify quantitative loci (QTL) for several characteristics, including resistance to diseases. For rapid mapping of monogenic resistance genes in segregating populations, bulked segregant analysis (BSA) procedure is followed frequently. Location of many disease resistance genes in clusters in the genome has been revealed by employing molecular markers. Some of the molecular markers linked to disease resistance genes are presented in Table 3.2.

In the recent years, development of microarray-based expression profiling methods, in addition to the availability of genomic and/or expressed sequence tag (EST) data for some plant species has paved the way for significant progress in characterization of plant pathogenesis-related responses. It is possible to monitor the expression of hundreds or thousands of genes simultaneously, by applying DNA microarray technology. New pathogenesis-related genes, co-regulated genes and the associated regulatory systems can be identified. Two types of DNA microarrays, viz., cDNA microarrays and oligonucleotide-based arrays are commonly used to study plant-pathogen interactions. DNA microarray technique allows the

Table 3.2 Molecular markers linked to resistance genes in different pathosystems

Crop/Pathogen	Resistance gene	Type of markers*	References
Apple/*Podosphaera leucotricha*	QTLs on LGs 2 and 8	RGAs	Calenge and Durel (2006)
Barley/*Erysiphe graminis* f.sp. *hordei*	*Ml (La)*	RFLP	Giese et al. (1993)
Bean/*Colletotrichum lindemuthianum*	*Co4*	RAPD	Cardoso de Arruda et al. (2000)
	R genes on *B*11	RGAP	Mutlu et al. (2006)
Grapevine/*Uncinula necator*		RFLP	Reisch (1998)
Lettuce/*Bremia lactucae*	*Dm17* and *18*	RAPD, SCAR	Maisonneuve et al. (1994)
Maize/*Bipolaris maydis*	*rhm*	RFLP	Zaitlin et al. (1993)
Potato/*Phytophthora infestans*	*R1* and *R3*	RFLP	El-Kharbotly et al. (1994)
	Ph3	AFLP	Chungwongse et al. (2002)
Rice/*Magnaporthe grisea*	*Pi-z locus*	SSR	Fjellstrom et al. (2006)
Soybean/*Phytophthora sojae*	*Rps 1-k*	RAPD	Kasuga et al. (1997)
Wheat/*Fusarium graminearum*	QTL on *1B* and *3B* chromosomes	AFLP and SSR	Zhang et al. (2004)
Wheat/*Puccinia recondita*	*Lr 9*	RAPD	Schachermayr et al. (1994)
Yam/*Colletotrichum gloeosporioides*	*Dcg-1*	RAPD	Mignowna et al. (2002)

* AFLP – amplified fragment length polymorphism; RAPD – random amplified polymorphic DNA; RFLP – random amplified fragment length polymorphism; RGA – resistance gene analogs; RGAP – resistance gene analog polymorphism; SCAR – sequence characterized amplified region; SSR – simple sequence repeat.

researchers to examine the responses of hundreds or thousands of genes simultaneously during a given treatment. Based on these expression profiles, it is possible to identify the differentially present mRNA species, based on which potential defense-associated functions may be inferred. Hypothesized *R* gene products are considered to have significant role in initiating many plant defense responses. However, DNA microarray experiments indicated that most of the known *R* genes did not exhibit high levels of transcriptional regulation. Possibly many *R* genes do not need to be regulated, but instead encode the constitutive receptors that regulate the expression of other genes. Alternatively, *R* gene expression may be required at post-transcriptional levels (Austin et al. 2002; Nishimura and Somerville 2002). It can be expected that using DNA microarray technology, any new defense-related genes may be identified in plants, as more genomes and ESTs are sequenced and as DNA microarrays become less expensive and more accessible to researchers (Wan et al. 2002). The genetic bases of resistance to diseases caused by microbial pathogens in some economically important crops are discussed below:

Wheat

Puccinia graminis f.sp. *tritici* causing wheat stem rust disease exists in the form of large number of races with different virulence genes capable of adapting to the cultivars with resistance conferred by simple major genes. Because of the development of new races with virulence genes compatible with resistance genes of newly developed wheat cultivars, continuous efforts were required to replace the 'defeated' resistance genes. In order to enhance the effectiveness of major gene resistance by reducing the rate of pathogen proliferation, many different approaches were made. The ability of wheat cultivars such as Thatcher and New Thatcher to retard stem rust disease development was recognized. The exhibited resistance to the disease was measured by infection types and slow development of rust pustules. Wilcoxson et al. (1975) showed that cvs. Exchange, Thatcher, McMurachy, Redman, Keny 58, Frontana and Idaed retarded rust development more effectively than other cultivars tested, based on the disease assessment using the area under disease progress curve (AUDPC) procedures. Slow disease development may be due to vertical or horizontal resistance of the cultivar concerned. Vanderplank (1984) suggested exploitation of this form of resistance, since heritability of resistance is high (90% or more) and the resistance is conditioned by a relatively few genes conferring resistance additively.

Wheat stem rust has been effectively controlled through the use of resistant cultivars in wheat for over 50 years, in combination with eradication of the alternate host common barberry (*Berberis vulgaris*). The stem rust resistance gene *Sr31* derived from Petkus rye has been incorporated into wheat varieties grown in various countries. The gene *Sr31* was the main component of stem rust resistance and remained effective until recently, when isolates of *P. graminis* f.sp. *tritici* (TTKS) with virulence to *Sr31* were detected in Uganda in 1999 (Pretorius et al. 2000). The race TTKS may pose a threat to wheat production, especially in developing countries where *Sr31* has been used as the primary component for stem rust resistance in commercial wheat varieties. However, resistance to race TTKS has been detected in major classes of wheat in the US (Jin and Singh 2006). Singh and Gupta (1992) first identified *Lr34,* the gene conferring slow-rusting resistance to Mexican races of *P. triticina* (*P. recondita* f.sp. *tritici*) in wheat cv. Pavon 76. Another slow-rusting resistance gene *Lr46* located in the chromosome 1B of Pavon 76 was identified in a later study (Singh et al. 1998). The CIMMYT wheat lines with *Lr34* gene complex showed slow-rusting resistance which was attributed to 3–4 additive resistance genes and this resistance was durable (Dubin and Rajaram 1996).

The disease intensity assessment by AUDPC has been found to be effective to characterize foliar disease resistance, since it reflects both severity and rate of disease development (Jeger and Vijanen-Rollinson 2001). Genetic studies revealed that slow leaf-rusting resistance is under polygenic control with moderately high heritability (Das et al. 1992). Of the two genes *Lr34* and *Lr46*, the former has been widely used in wheat breeding programs, because of its durable resistance to leaf rust and its association with *Yr18*, a stripe rust resistance gene, in addition to tolerance to *Barley yellow dwarf virus* infection (Mc Intosh 1992; Singh 1993). The

combination of *Lr34* with other rust resistance genes such as *Lr12* and/or *Lr13* resulted in production of several leaf rust resistant cultivars worldwide (Roelfs 1988).

Three RAPD markers associated with leaf rust resistance gene *Lr34* were identified using bulked segregant analysis (BSA), two of them being located on 7BL and the third one hybridizing to chromosome 1BS and 1DS (William et al. 1997). Six QTLs for leaf rust resistance and one major QTL on 7BL from the highly resistant parent Forno were also detected (Messmer et al. 2000). Later Schnursch et al. (2004) identified eight QTLs for leaf rust resistance with two having major effects. In addition, two AFLP markers linked to *Lr46* and another resistance gene were identified and they were located on the distal end of the long arm of chromosome 1B (Williams et al. 2003). The genotype CI13227 was reported to offer the highest level of slow leaf-rusting resistance (Shaner et al. 1997). By using molecular markers, the slow leaf rusting resistance conferred by CI13227 was identified. Four hundred and fifty nine AFLP markers and 28 simple sequence repeat (SSR) markers were analysed in the population. Two QTLs named as *QLr.osu-2B* and *QLr.osu-7BL* were found to be consistently associated with AUDPC, final severity (FS) and infection rate (IR) of leaf rust pathogen. Both the QTL and correlation analysis indicated reasonable progress in leaf-rusting resistance by selecting for final severity. The results revealed that SSR markers closely associated with *QLr.osu-2B* or *QLr.osu-7BL* have the potential for use in MAS for durable leaf rust resistance cultivars (Xu et al. 2005).

Map-based cloning and functional genetic studies in model plant systems have been possible with the availability of whole-genome sequences and provide fundamental knowledge for understanding plant growth and environmental response including pathogen infection. The *Lr21* gene, conferring resistance to leaf rust disease, was first incorporated into wheat cv. Thatcher from *Aegilops tauschii* accession TA1599 via synthetic wheat (Rowland and Kerber 1974). No virulent isolate of *P. triticina* was reported to break *Lr21* resistance. The leaf rust resistant germplasm lines WGRC2 and WGRC7 were developed by introgressing an *Lr21* allele (earlier designated as *Lr40)* from a different accession (TA1649) (Raupp et al. 1983; Huang and Gill 2001). The map-based cloning of the gene *Lr21* from the large genome of bread wheat lines WGRC2 and WGRC7 introduced a high degree of polymorphism in the region flanking the gene and expedited mapping of markers at the desired genetic locus. This study demonstrates that map-based cloning is a viable strategy for accessing genes from the large polyploid genome of bread wheat. Cloning of *Lr21* can facilitate studies on gene organization, evolution and host-pathogen interaction to elucidate the molecular mechanism of resistance in a polyploid wheat model (Huang et al. 2003).

Virulence shifts in *P. triticina* populations have made the existing the leaf rust resistance genes less effective in protecting wheat plants. The gene *Lr16* is a leaf rust gene conferring resistance at the seedling stage. However, the frequency of virulence to *Lr16* has appreciably increased during the past decade. The approach of pyramiding resistance genes to enhance the level of resistance of genotypes has been tested for feasibility. Pyramiding *Lr16* with other leaf rust resistance genes may be achieved by the use of molecular markers linked to resistance genes for

marker-assisted selection (MAS). In order to identify markers linked to *Lr16* suitable for MAS were identified by tagging *Lr16* with microsatellite markers on the distal end of chromosome 2BS in three mapping populations. Of the seven microsatellite loci mapped to *Lr16*, *Xwmc 764* was the closest microsatellite locus. Three microsatellite markers Xwmc764, Xgwm 210 and Xwmc 661 were selected as the most suitable ones, since they had simple PCR profiles, numerous alleles and high polymorphism information content (PIC). Further, they were tightly linked to *Lr16*. The results of leaf rust resistance evaluation based on infection type (IT) data and the microsatellite allele data were in good agreement. Furthermore, microsatellite markers were shown to be useful for postulating *Lr16* in wheat lines with multiple leaf rust resistance genes (Mc Cartney et al. 2005).

Powdery mildew caused by *Blumeria* (*Erysiphe*) *graminis* f.sp. *tritici* is another important disease causing considerable yield loss in wheat crops. As in the case of wheat rust disease, use of resistant wheat cultivars is the preferred disease management strategy. Thirty two *Pm* genes (*Pm1* to *Pm32*) conferring resistance to this obligate biotrophic pathogen have been genetically characterized (Hsam and Zeller 2002; Mohler et al. 2006). The *Pm3* was one of the first described loci and 10 different resistance specificities (*Pm3a* to *Pm3j*) have been located to the *Pm3* locus on the short arm of wheat chromosome 1A (Briggle and Sears 1966; Zeller and Hsam 1998). The resistant genes, identified by classical genetic studies and molecular studies of cloned genes, are placed in two distinct genomic arrangements. The gene *Pm3b* isolated from the hexaploid (*Triticum aestivum*) bread wheat genome encodes a CC-NBS-LRR protein and belongs to a large family of resistance gene-like (RGL) genes spread over approximately 1Mb on the short arm of chromosome 1A (Yahiaoui et al. 2004). By using molecular markers (RFLP and microsatellite markers) closely linked to *Pm3b,* haplotype analysis of 10 lines carrying different *Pm3* alleles was performed. All these lines showed the presence of a conserved genomic region delimited by markers cosegregating with *Pm3b* and including a structurally conserved *Pm3b*-like gene. A *Pm3b*-like sequence from lines carrying *Pm3a, Pm3d* and *Pm3f* alleles was amplified by a PCR-based strategy (Srichumpa et al. 2005). No avirulence gene has been isolated from wheat powdery mildew pathogen, as in the case of flax rust pathogen *Melampsora lini* in which an avirulence gene products have been cloned and demonstrated to be expressed in rust haustoria (Dodds et al. 2004).

The evolution of the complete set of *Pm3* resistance alleles was analysed. The *Pm3* bread wheat resistance alleles might have evolved recently from a common ancestral gene template, as indicated by the low level of their sequence diversity. The ancestral gene was identified as a susceptible allele *Pm3CS* from which *Pm3* resistant alleles were generated. The *Pm3CS* is widespread among hexaploid bread wheat lines and it showed identity (from 97 to 99%) to the *Pm3* resistance alleles. Results from functional analysis demonstated that *Pm3* recognition specificities were determined by a limited number of amino acid polymorphism between *Pm3* alleles. The presence of the *Pm3CS* allele was detected in the wild tetraploid wheat, the ancestor of hexploid bread wheat. The wild tetraploid wheat was located in southern Turkey, a possible site of wheat domestication. The *Pm3* and *Lr10* loci

in wheat may represent two different types of *R*-gene evolution reflecting different forms of interaction with their respective avirulence gene products. So far the *avr* genes have not been identified in both biotrophic pathogens affecting wheat. In order to have an insight into the nature of the interactions between these pathogens and wheat, the *avr* genes have to be isolated and characterized (Yahiaoui et al. 2006).

Triticum dicoccoides has the potential to be used as a valuable genetic resource for disease resistance genes. The *T. dicoccoides*-derived wheat line Zecoi-1 provides effective protection against powdery mildew. The resistance in line Zecoi-1 has been shown to be controlled by a single dominant gene. Eight markers were identified based on AFLP analysis of bulked segregants from F_3 populations of the cross between Chinese Spring X Zecoi-1. The AFLP markers were mapped in the wheat chromosome 2BL. Based on disease response patterns, genomic origin and chromosomal location, the resistance gene in Zecoi-1 was tentatively designated *Mlec1*. Due to the ease of hybridization with, and introgression into, common wheat, it is expected that the contribution of *T. dicoccoides* to genetic improvement of wheat will be significant (Mohler et al. 2006).

Fusarium head blight (FHB) disease and its pathogen *Fusarium graminearum* are important, because of the direct economic losses to the wheat crop and mycotoxicoses induced in humans and animals following consumption of grains contaminated with mycotoxins produced by the pathogen. Resistance to FHB has been classified into five different types: (1) resistance to initial infection, (2) resistance to spread of infection within a spike, (3) decomposition or non-accumulation of mycotoxins, (4) resistance to kernel infection and (5) yield tolerance (Mesterhazy 1995). Progress in breeding FHB resistant cultivars has been hampered by the nonavailability of effective sources of resistance and the complex nature of resistance to wheat diseases (Oliver et al. 2004). In addition, selection based on visual symptoms may not provide reliable identification of resistance sources at flowering when expression of resistance genes is markedly affected by temperature and humidity. Evaluation of a large number of accessions (1507) of 93 species from 18 genera under *Triticeae*, resulted in the identification of 31 accessions with high levels of Type I resistance and 151 acessions with high levels of Type II resistance (Wan et al. 1997). The native Japanese species *Elymus humidus* was found to be immune to FHB (Fedak 2000). FHB resistance in some of the wild species has been transferred to wheat by producing alien chromosome addition, substitution and translocation lines. RFLP analysis showed that two of three *Leymus racemosus* chromosomes conferring resistance to FHB belonged to homologous groups 5 and 7 (Qi et al. 1997). Resistance in *L. racemosus* was governed by multiple genes located on at least three chromosomes (Chen and Liu 2000).

Sumai 3 and its derivatives like Ning 7840 have been used extensively as sources of resistance and the FHB resistance genes from these genotypes have been well characterized through molecular mapping (Bai et al. 1999; Zhou et al. 2002; Bai et al. 2003). Wangshuibai a chinese landrace possesses high level of resistance to fungal spread within a spike and the resistance was found to be more stable than that of Sumai 3 (Lu et al. 2001). The recombinant inbred lines (RILs) derived from the cross between susceptible cv. Alondra and Wangshuibai were assessed. Fifteen

AFLP markers associated with three QTL for FHB resistance were located on two chromosomes. One QTL was mapped on 1B and two others were mapped on 3B. One QTL on 3BS had a major effect. The MAS for FHB resistance genotypes can be made using closely linked markers like SSR markers. It is possible to eliminate undesirable associations between FHB resistance and other agronomic traits (Zhang et al. 2004). By making crosses between wheat and rye, common cultivars and breeding lines with FHB resistance have been developed (Lu et al. 2000; Oliver et al. 2004). Wheat-alien species derivatives (7300) were screened for Type II FHB resistance. Some of the resistant derivatives showed comparable level of resistance found in Sumai 3 (Oliver et al. 2004). It was suggested that pyramiding of the alien resistance genes in wheat may be an effective approach to enhance the level of resistance to FHB that may be durable (Cai et al. 2005).

Rice

Among the fungal pathogens infecting rice crop, *Magnaporthe grisea* (anamorph-*Pyricularia oryzae*) causing blast disease occurs in all rice-growing countries accounting for significant losses. About 40 blast resistance loci have been identified and mapped in rice. More than 20 loci segregate as single dominant genes, whereas 10 loci are associated with quantitiative resistance (Chen et al. 2004). Earlier two resistance genes viz., *Pi-b* and *Pi-ta* had been cloned and characterized. The *Pi-b* gene belongs to NBS-LRR class *R* genes. On the other hand, the *Pi-ta* gene encodes a protein containing an NBS domain and leucine-rich domain (LRD), a domain corresponding to the LRR of the RPM1 protein (Wang et al. 1999b; Bryan et al. 2000).

The Chinese native cultivar Q14 was found to be resistant to many isolates of *P. grisea* (*Magnaporthe grisea*) existing in Japan, Thailand and China. A linkage analysis using microsatellite markers was performed on the F(2) population of the cross between Q14 and susceptible *indica* cv. Q61 through bulked segregant analysis (BSA) in combination with recessive-class analysis (RCA). Two markers RM151 and RM 259 located on chromosome showed positive and negative polymorphisms, respectively, for a resistance gene segregating in the population. The new resistance gene identified in this investigation was designated tentatively as *Pi27(t)* (Zhu et al. 2004). The gene-for-gene interaction between rice plant and *M. grisea* has been exemplified by race-cultivar specificity which reveals that the pathogen carries a gene for avirulence (*avr*) that corresponds to each resistance gene in the host (Silué et al. 1992a). An increasing number of avirulence genes in *M. grisea* corresponding to more than 40 major rice blast resistance loci (*Pi* genes) have been analysed (Notteghem et al. 1994; Sallaud et al. 2003). Four avirulence genes *PWL2, AVR1-CO39, AVR-Pita* and *ACE1* have been isolated and characterized. The *Avr-Hattan3* was shown to correspond to a resistance gene at *Pik* locus which is known to have several blast resistance genes. Random amplified polymorphic DNA (RAPD) markers and restriction fragment length polymorphism (RFLP) markers from genetic maps of *M. grisea* were used to construct a partial genetic map of *Avr-Hattan 3* (Yasuda et al. 2005).

Molecular markers from cloned genes have been developed for MAS in rice breeding for disease resistance (Jia et al. 2002). Digu, a Chinese *indica* (*Oryza sativa* subsp. *Indica*) variety showed durable resistance to all 156 isolates of *M. grisea* collected from China and Japan. The genetic basis of blast resistance in Digu to the two Chinese isolates ZB13 and ZB15 of *M. grisea* that caused severe blast was studied. A novel strategy combining resistance gene analog (RGA) assay and uneven PCR were employed to assess the identities of new genes that were different from the known blast resistance genes and to tag the new resistance genes with molecular markers. Resistance of Digu to isolates ZB13 and ZB15 were controlled by two different single dominant genes. As these two genes were different from the known blast resistance genes, they were named as *Pi-d (t)1* and *Pi-d(t)2* which were respectively located in chromosome 2 and 6. With tagging of these genes using RGA assay and uneven PCR technique, two RGA markers, SPO 01 and SPO 03 that were co-segregated to *Pi-d(t) 1* and *Pi-d(t) 2* respectively, were identified (Chen et al. 2004). In a further study, the *Pi-d2* (earlier named *Pi-d(t)2*) was isolated by a map-based cloning strategy. The *Pi-d2* encoded a receptor-like kinase (RLK) protein with a predicted extracellular domain of a bulb-type mannose-specific binding lectin (ß-lectin) and an intracellular serine-threonine kinase domain. Transgenic plants carrying the candidate *Pi-d2* gene (either genomic or the cDNA) showed a race-specific resistance to the *M. grisea* strain ZB15. The *Pi-d2* protein was plasma membrane localized. The resistant and susceptible alleles of rice blast resistance gene *Pi-d2* were differentiated by a single amino acid difference at position 441 of Pi-d2 protein. The *Pi-d2* gene represents a new class of plant resistance genes, because of the presence of a novel extracellular domain (Chen et al. 2006a).

The *Pi-z* gene in cv. Zenith is a broad spectrum *Pi* gene widely used by researchers in several countries. The *Pi-z* was mapped on rice chromosome 6 and found to be allelic to the *Pi2(t)* resistance gene (Inukai et al. 1994). DNA markers have been useful to incorporate blast resistance factors at the *Pi-z* locus into improved cultivars using MAS procedure. The markers used earlier included one based on restriction enzyme digestion of PCR amplification products being linked to the *Pi2(t)* gene (Hittalmani et al. 1995), dominant PCR markers based on resistance gene analog sequences and codominant simple sequence repeat (SSR or called microsatellite) markers linked to the *Pi-z* gene (Conaway-Bormans et al. 2003) and single nucleotide polymorphism (SNP) markers linked to the *Pi-z and Pi-z*^{t} genes (Hayashi et al. 2004). In a later study, SSR markers more tightly linked to and flanking both sides of *Pi-z* locus were developed. Three SSRs on rice PAC AP005659 were found to be very tightly linked to the *Pi-z* locus, with one marker AP5659-3, co-segregating with *Pi-z* resistance reaction. Two SSR marker haplotypes were found to be unique for cultivars carrying the *Pi-z* gene which indicated that these markers could be advantageously employed for selection of resistance genes at the *Pi-z* locus in rice germplasm. In addition, these markers could be used for selection of the *Pi2(t), Pi9(t), or Pi-Z*^{t} resistance genes, as these are alleles present at the same locus (Fjellstrom et al. 2006).

Single nucleotide polymorphisms (SNPs) have been shown to be an useful molecular breeding tool. Low cost, simple and throughput method of detection of

SNPs is essential for using them as molecular markers. A real-time PCR using different probe detection systems have been applied. TaqMan MGB probes have been found to have improved performance for hybridization probe-based SNP typing. Locked nucleic acid (LNA) modified displacement probe for the SNP responsible for the amino acid change in the *Pi-ta* gene was designed for rapid genotyping the *Pi-ta* SNP in a range of rice cultivars. The high specificity of the LNA displacement probe assay made it possible to perform the assay on genomic DNA extracted from pooled leaf samples with a 9:1 ratio of either susceptible to resistant or resistant to susceptible genotype. This assay was more efficient in accurately identifying the presence of low frequency genotypes in artificially created mixed samples. The SNP responsible for blast resistance conferred by the *Pi-ta* gene was accurately identified, making this assay ideal for MAS in breeding programs targeting this important blast disease resistance gene. This assay is simple, rapid and has the potential to analyze pools of upto 10 samples resulting in considerable reduction in the cost of the testing (Kennedy et al. 2006).

In another investigation, the *Pi2* gene was identified using a map-based cloning strategy. The gene cluster including *Pi2* gene, comprises nine members (*Nbs1–Pi2* to *Nbs9–Pi2*). *PiZ* encodes a protein with a NBS-LRR domain. The results of fine genetic mapping, molecular characterization of the *Pi2* susceptible mutants and complementation tests showed that *Nbs4–Pi2* was the *PiZ* gene. Likewise, the family member *Nbs4–Piz-t* was *Piz-t*. The sequence comparison revealed that only eight amino acid changes which were confined within three consecutive LRR, differentiated *Piz-t* from *Pi2*. A reciprocal exchange of the single amino acid between *Pi2* and *Piz-t* did not change the resistance specificity to each other. But such a change rather abolished the function of both resistance proteins (Zhou et al. 2006a).

Apple

Apple scab caused by *Venturia inaequalis* is one of the most serious diseases of apple in all the countries worldwide. Development of cultivars with built-in resistance is the desirable approach, since chemical control of the disease is expenssive and environmentally unsuitable. Breeding for resistance to apple scab has been slow, because apples have self-incompatibility and a long juvenile period. Identification of molecular markers linked to apple scab resistance can be expected to accelerate breeding by making it possible to select resistant individuals, regardless of growth stage, interaction between resistance genes, source of inoculum and environmental condition. The marker should be either functionally responsible for resistance or tightly linked to resistance to scab disease. Scab resistance encoded by *Vf* gene was discovered in the small-fruited wild crab apple *Malus floribunda* clone 821 which has been the most widely used source (Vinatzer et al. 2001). *Vf* gene has been incorporated into more than 70 apple cultivars showing resistance to apple scab (Janick et al. 1996). The other genes conferring resistance to scab are *Vr* from *M. pumila*, *Vm* from *M. micromalus* and *Va* from *Malus* hybrid 'Antonovka' (McHardy 1996).

A cluster of receptor-like genes at the *Vf* locus from the *Vf* containing M x *domestica* cv. Florida was cloned and they were deisngated *Hcr Vf-1, HcrVf-2, HcrVf-3* and *Hcr Vf-4*. These genes showed high similarity ($\geq$ 90%) in most of their domains, but showed variation in the LRR domain (Vinatzer et al. 2001). In a later study, four similar genes from the *Vf* locus of *M. floribunda* 821 and *M* x *domestica* cv. Goldrush were cloned and named as *Vfa1, Vfa2, Vfa3* and *Vfa4* (Xu and Korban 2002). A wide range of apple cultivars and lines were compared to identify which one of the described *Vf* genes was most closely linked to *Vf* resistance. Primers were designed based on the conserved regions in the *Vf* candidate genes adjacent to variable portion of the LRR domain to yield PCR product length polymorphisms. PCR products from 31 cultivars of *M* x *domestica,* of which 19 showed *Vf* resistance and from 10 selections of *M. floribunda.* PCR products (484 and 646 bp) corresponding to *Vfa1* and *Vfa2* were present in all plants tested. In contrast, a PCR product (286 bp) with 100% predicted amino acid identity to *Vfa4* was detected only in *M* x *domestica* cultivars with *Vf* scab resistance. The sequence of the 286 bp PCR product most closely matched that of *Vaf4*. This study confirmed that a 286 bp PCR product with a sequence matching *Vfa4* gene was the most reliable molecular marker for the detection of *Vf* scab resistance in apple (Afunian et al. 2004).

Although the several scab resistant apple cultivars with *Vf* resistance have been released, the appearance of the apple scab pathogen races 6 and 7 that could overcome *Vf* resistance necessitated to widen the genetic basis of scab resistance. Attempt was made to analyze the population genetic process within the matching virulent sub-population of *V. inaequalis* following the break down of *Vf* resistance. The AFLP procedure and allelic variation at four microsatellite loci were applied to determine the genetic structure of 133 isolates of *V. inaequalis* from a single commercial apple orchard sampled from one cultivar carrying the *Vf* gene and three cultivars without *Vf* gene. A strong decrease of the genetic diversity among isolates from the *Vf* cultivar, and high level of diversity among isolates from three other apple cultivars were revealed by both analysis. A high genetic differentiation between *Vir Vf* and *avr Vf* groups was seen. All *VirVf* isolates could be placed to a single clonal lineage (Guérin and Le Cam 2004).

It is possible to achieve durable resistance by pyramiding several major resistance genes in the same background. Cultivars with different resistance genes may be crossed and with the help of suitable molecular markers associated the resistance genes, plants with desired allele combination can be selected by MAS procedure. Five loci conferring qualitative apple scab resistance, in addition to *Vf* have been identified. The locus *Vbj* is associated with the SSR CHO5eO3 (Gygax 2004). While *Vr* locus is associated with SSR (CHO2b10 and the RAPD marker $OPB18_{620}$, the *Vx* locus is SCAR marker $S22_{1300}$ (Hemmat et al. 2002). The loci *Vf* from Hansen's Baccata No. 2 and *Va* from PI 172623 were earlier identified (Williams and Kuć 1969; Lespinasse 1989). Another resistance gene was located in GMAL 2473 (Russian seedling 2). By applying bulked segregant analysis (BSA) of three AFLP markers and one RAPD marker associated with the GMAL 2473 resistance gene was identified and named as *Vr2*. It is now possible to develop selections carrying

a new major apple scab resistance gene *Vr2* by using its molecular markers. New generations of cultivars carrying several apple scab resistance genes in the same background can be obtained through molecular marker-assisted selection (MAS) procedure (Patocchi et al. 2004).

Another important disease seriously damaging apple is powdery mildew caused by *Podosphaera leucotricha*. Resistance to powdery mildew is derived from two main sources namely (i) weak to strong polygenic resistance from *Malus* × *domestica* and (ii) monogenic resistances generally derived from wild related species or ornamental crab apples. Five major genes viz., *Pl-1, Pl-2, Pl-w, Pl-d* and *Pl-m* have been identified (Alston et al. 2000). To avoid the risk-associated monogenic resistance and weak polygenic resistance, combination of monogenic and polygenic resistances may be an effective approach to offer multiple resistance barriers against the powdery mildew pathogen. Molecular markers to detect the presence of major resistance genes are available. However, molecular measures for polygenic resistance are yet to be identified. The identification and mapping of the genes governing polygenic resistance (quantitative trait loci) has to be carried out. A QTL analysis was performed in the F_1 progeny of cross between cv. Discovery and hybrid TN10-8. At least 4 or 5 QTLs according to the season were identified. Some of the QTLs (LGs2 and 8) identified co-localized with major powdery mildew or scab resistance genes or NBS-LRR resistance gene analogues identified in the same progeny earlier. The stable QTLs identified on LGs 2 and 13 together with the strong effect of QTL located on LG8 are considered to be of special interest for breeding programs (Calenge et al. 2005; Calenge and Durel 2006).

Potato

Disease resistance in potatoes may be governed by genes at one or few loci (monognic, oligogenic or major gene resistance encoded by *R* genes). Resistance conferred by the *R* genes may become ineffective when new races or biotypes or strains compatible with the *R* gene(s) are produced. The need for differentiation of host resistance was first suggested by Vanderplank (1963). Most of the *R* genes conferring resistance to *Phytophthora infestans* causing the late blight disease were identified in *Solanum demissum* (Malcolmson and Black 1966). The pathogen appeared to be capable of producing new races at a faster rate than scientists could produce new potato varieties with unmatched resistance genes. A shift in the objective of breeding became essential and breeding for potato cultivar with polygenic or non-specific resistance (field resistance) was considered to be an effective alternative (Holden 1977). Molecular techniques have been shown to be useful for characterization of different stages of interaction between the host plant species and microbial pathogens. By employing molecular markers, all regions of the plant genome can be assayed for linkage of disease resistance. Mapping of the genome using molecular markers may help in identifying QTLs for several characteristics including disease resistance.

In potato, a high-resolution genetic map at the *R*3 locus conferring high resistance to avirulent isolates of *P. infestans* was constructed. The *R*3 locus consists

of two genes, *R3a* and *R3b*, with distinct specificities which were 0.4cM apart. These genes from *S. demissum* were incorporated into potato. One accession of *S. demissum* had a natural recombination between *R3a* and *R3b* (Huang et al. 2004). A new late-blight resistance (*R*) locus in *Solanum bulbocastanum* was identified. A high-resolution genetic map of the new locus was generated, delimiting *Rpi-blb3* to a 0.93cM interval on chromosome 4. An AFLP marker that cosegregated with *Rpi-blb3* in progeny plants (1396) of an intraspecific mapping population was identified. In addition, three other late-blight resistance genes, *Rpi-abpt 2, R2* and *R2*-like appear to reside in the same R gene cluster on chromosome 4 as the newly identified gene, *Rpi-blb3* (Park et al. 2004).

The DNA markers (30) from tetraploid potatoes were tested for linkage with QTLs for late blight resistance using PCR assay. Most of the markers were shown to originate from within, or be physically closely linked to, candidates for quantitative resistance factors. Assessment of interactions between unlinked QTL for resistance may lead to identification of testable strategies for MAS (Bormann et al. 2004). The quantitative resistance to *P. infestans* may be linked to undesirable agronomic traits. For example, the potato cultivars (600) bred between 1950 and 1990 in a gene bank collection were genotyped with five DNA markers linked to previously mapped QTL for resistance to late blight and plant maturity. By using PCR markers specific for *R1*, a QTL for disease resistance and late maturity was identified. The marker alleles associated with greater resistance were traced to *S. demissum* as donor of resistance genes (Gebhardt et al. 2004).

An in vitro inoculation procedure was developed using potato plantlets for monitoring major *R* genes in potato and their corresponding *Avr* genes in *P. infestans*. A population of 93 clones was phenotyped for segregation of two closely linked and functionally distinct genes-*R3a* and *R3b*-in the *R3* locus. Phenotyping of the population was fully consistent with genotyping obtained from analysis of molecular markers that flank each gene. The in vitro assay was found to be a reliable alternative to detached leaf assay to phenotype a large population segregating in the *R3* locus. The in vitro assay was effective in phenotyping the primary transformants of the *R3a* gene candidates, resulting in the identification of the *R3a* gene at least 2 months earlier than the detached leaf assay. A perfect correlation between the results obtained from these two assays, indicated that the in vitro inoculation method may be preferable, because of its rapidity, space-effectiveness and accuracy (Huang et al. 2005).

Common Bean

Common bean (*Phaseolus vulgaris*) is severely affected by the anthracnose disease caused by *Colletrotrichum lindemuthianum.* High genetic variability of this pathogen is reflected in the existence of various races characterized based on phenotypic reaction on a set of 12 universal differential cultivars. Ten genes (*Co-1* to *Co-10)* governing resistance to different sets of pathogens races have been identified (Pastor-Corrales 1991). Allelism tests have indicated that *Co-1* to *Co-6* are located at independent loci. In a later study, allelism tests and molecular analysis

were combined to characterize the genes for resistance using germplasm lines A321 and A493. RAPD markers $OB12_{350}$ $OAH18_{1100}$, and $OY17_{1100}$ and SCAR markers SI19 and SW12 were found to be linked to the resistance gene. The results suggest that *Co-3* and *Co-9* considered as different genes earlier, are infact different '*R*' gene combinations (alleles) of the *B*4 resistance gene cluster. Five different alleles at this cluster were identified. Multiple loci tend to complicate the conclusion from studies using different pathogen races (Méndez-Vigo et al. 2005).

Polymerase chain reaction (PCR)-based resistance gene analog polymorphism (RGAP) markers were identified for mapping candidate genes and QTL for disease resistance in common bean. A PCR approach with degenerate primers based on two conserved motifs of NBS-LRR type plant *R*-genes was applied to develop RGAP markers. The markers mapped to 10 of 11 linkage groups with a strong tendency for clustering. Further, RGAP markers co-located on six linkage groups, with 15 resistance gene analogs (RGAs) that were previously mapped in other populations of common bean. A common mechanism seems to exist between *R*-genes and QTL for resistance in common bean since RGAP markers colocalized with QTLs for resistance to different diseases on B2, B6, B8, B10 and B11. Rust resistance genes and an anthracnose resistance gene were found to flank seven RGAP markers on B11. RGAP markers appear to be better candidates for locating genomic regions associated with disease resistance compared with random DNA markers. PCR-based RGAP mapping is much less expensive and faster than RGA mapping because RGAs require cloning and RFLP mapping (Mutlu et al. 2006). Allelism tests were performed to study the relationship among identified genes conferring resistance to *Phaeoisariopsis griseola* causing angular leaf spot in common bean. Allelic forms for the resistance genes in four resistance sources were determined (Caixeta et al. 2005).

Lettuce

Clustering of resistance genes in the genomes of several crop plant speices have been demonstrated by classical genetics. In lettuce (*Lactuca sativa*) majority of known resistance specificities to seven diseases are clustered in five linkage groups (Bonnier et al. 1994; Witsenboer et al. 1995). The presence of conserved domains in resistance gene has been exploited to clone numerous additional resistance genes from diverse species by employing PCR assay with degenerate oligonucleotide primers to conserved motifs. The resistance gene candidates (RGCs) from lettuce were amplified using multiple combinations of primers with low degeneracy designed from motifs in the nucleotide binding sites (NBSs) of *Arabidopsis thaliana* and *N* gene of tobacco. The relationship of the amplified products to resistance genes was evaluated by several sequence and genomic criteria. The genetic analysis demonstrated the existence of clustered multigene families for each of the four RGC sequences, indicating a parallelism between the classical and molecular studies. Fluorescent in situ hybridization revealed the clustered genomic distribution of RGC sequences. This study indicated the suitability of using PCR assay with degenerate

oligonucleotide primers as an efficient procedure for identifying several RGC in different crop plant species (Shen et al. 1998).

Introgression of resistance genes to lettuce has been attempted to improve level of resistance to downy mildew disease caused by *Bremia lactucae. Lactuca serriola* and *L. saligna* have been used as the donors of disease resistance gene(s) (Crute 1992). The *RGC2* locus in lettuce is one of the largest clusters of resistance gene candidates (RGCs). At least eight *Dm* genes for resistance to downy mildew have been mapped to this region (Kesseli et al. 1994). The gene *Dm3* (*RGC2B*) a member of *RGC2* family was shown to be necessary and sufficient to confer resistance to isolates of *B. lactucae* that expressed the *Avr3* avirulence gene (Shen et al. 2002a). A large number of *RGC2* sequences encoding fragments of the LRR region from seven genotypes of three *Lactuca* spp. was compared to understand the genetic events occurring at the *RGC2* locus in lettuce. Two distinct types of *RGC2* genes (Type I and Type II) differing in their patterns of sequence divergence could be distinguished. Type I genes were extensive chimeras caused by frequent sequence exchanges, whereas type II genes exhibited occasional sequence exchange between paralogous sequences. The *RGC* 2 cluster was considered to be evolving rapidly in terms of its composition of Type I and Type II genes as well as the specific combination of LRRs encoded by Type I genes (Kuang et al. 2004).

3.1.2 Molecular Basis of Resistance to Fungal Diseases

Plants have two principal kinds of defense mechanisms. Passive or pre-existing defense mechanisms involving structural barriers such as waxy cuticle or reservoirs of antimicrobial compounds that are strategically positioned to act on the invading pathogens, are known to operate in some pathosystems. Active defense mechanisms are induced, when structural barriers are breached by the pathogens, resulting in prevention of further colonization of plant tissues. Active defense responses induced by fungal, bacterial and viral pathogens may be of three types viz., primary responses, secondary responses and systemically acquired responses. Primary responses are restricted to the infected cells or in close proximity with the pathogen and the specific signal molecules presented by the pathogens have to be recognized by the host cells. The outcome of this interaction may rapidly lead to the phenomenon known as programmed cell death (PCD). PCD is defined as a self-destruction process triggered by external or internal factors and it is mediated through an active genetic program which is considered to have a crucial role in the development and survival of diverse organisms (Pontier et al. 2004). Secondary responses are exhibited by the adjacent cells surrounding the initially infected cell in response to diffusible signal molecules (elicitors) released by pathogens. Systemically acquired response is due to the action of defense-related compounds that can be translocated throughout the plant, leading to systemic acquired resistance (SAR) (Hutcheson 1998).

The gene-for-gene hypothesis of Flor (1956) is based on the specific genetic interactions between host plant resistance (*R*) genes and the pathogen avirulence

(*Avr*) genes, as shown by flax–rust pathogen *Melampsora lini* interaction. The simple molecular interpretation of this plant-pathogen interaction is that *R* genes encode specific receptor site(s) for direct or indirect products of the corresponding *Avr* genes. The interaction between the gene products has been demonstrated using yeast-two hybrid system (Dodds et al. 2006). More than 30 flax rust resistance genes with different gene-for-gene specificities have been identified at five loci. Of these loci the *L* locus was particularly suitable for specificity studies, because it is a single gene with multiple alleles. *L, L1, L2 – L11*, encoding different resistance specificities. The *L*11 was a universally susceptible allele, conferring no identified resistance. The alleles encoded highly polymorphic proteins of the TIR-NBS-LRR (Toll interleukin-1 receptor homology – nucleotide binding site – leucine rich repeat) class. Of the L resistance proteins, L5 and L6 were among the most divergent, but capable of recognizing most of the same avirulence proteins encoded by alleles at flax rust pathogen, *Avr L 567* locus of rust strain CH5. The cloned *L1, L5 and L8 and L11* alleles were examined to confirm their function and specificity. One or more of the protein polymorphism may determine the specificity differences of the alleles (Ellis et al. 2007).

3.1.2.1 Gene-for-gene Interaction (Race-Specific Resistance)

Molecular mechanisms of host plants that differentiate 'self' and 'non-self' are fundamental in innate immunity to prevent potential infection by microbes. The studies on genetics of disease resistance have documented genetic polymorphism and recognition specificity and predicted that successful disease resistance can be triggered only if a resistance (*R*) gene product in the plant recognizes a specific avirulence (*Avr*) gene product of the pathogen. Many resistance (*R*) proteins that function as a surveillance system to detect pathogen AVR proteins (effectors) have been identified. Each AVR protein is considered to be detected by a specific R protein in gene-for-gene interaction which often triggers the hypersensitive response (HR). When an incompatible interaction is initiated, plants can activate a variety of inducible defense responses consisting of genetically programmed suicide of infected cells (HR) as well as tissue reinforcement and antibiotic production at the site of interaction. Following the local responses, a long lasting systemic response [systemic acquired resistance, (SAR)] is triggered, which in turn, primes the plant for resistance against a broad-spectrum of pathogens (Dong 2001; Métraux 2001). A substantial commitment of cellular responses including extensive reprogramming and metabolic reallocation will be needed for the multicomponent response of the host plant. This situation indicated that host plant defenses are kept under tight genetic control and activated as soon as the plant detects a prospective invader. Since plants lack a circulatory system, plant cells have to autonomously maintain constant vigilance against microbial pathogens by expressing large arrays of *R* genes (Dangl and Jones 2001).

The avirulence genes that have been cloned from fungal pathogens are relatively fewer in number compared to those of bacterial pathogens. Generally, the

bacterial plant pathogens synthesize effector proteins and deliver them into host cells where they are able to manipulate host defense including HR (Espinosa and Alfano 2004). On the other hand, fungal pathogens can deliver the effectors to both inside (cytoplasm) and outside (apoplast) of plant cells (Birch et al. 2006). Effectors are defined as molecules that manipulate host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defense responses (avirulence factors or elicitors) (Kamoun 2006).

The Avr proteins from extracellular fungal pathogens are secreted into the apoplast of plant tissues. These fungal effectors such as AVR2 (Luderer et al. 2002), AVR4 (Joosten et al. 1994) and AVR9 (van den Ackerveken et al. 1992) from *Cladosporium fulvum*, NIP1 from *Rhynchosporium secalis* (Rohe et al. 1995) and SIX1 from *Fusarium oxysporum* f.sp. *lycopersici* (Rep et al. 2004) are Cys-rich. The biochemical studies have shown that AVR4 is a chitin-binding protein that protects fungal cell walls from degradation by plant chitinases (Van den Burg et al. 2003) and AVR 2 is an inhibitor of the extracellular Cys protease Rcr 3 in tomato (Rooney et al. 2005). R proteins recognizing the *Cladosporium* extracellular Avr products are membrane-bound receptors with extracellular domains (Luderer et al. 2001). The *ACE1* Avr gene of *Magnaporthe grisea* encodes a hybrid polyketide synthase/nonribosomal peptide synthase (RKS-NRPS enzyme) involved in the biosynthesis of the effective avirulence signal (Böhnert et al. 2004). The Avr protein encoded by *Avr-Pi-ta* of *M. grisea* is predicted to encode a putative metalloprotease (Orbach et al. 2000). The interaction between AVR-Pita and the corresponding rice R protein Pi-ta is an example of direct interaction between R and AVR proteins (Jia et al. 2000). The nature of product of AVR gene *AVR-Co39* is yet to be determined (Farman et al. 2002). The *NIP1* gene in *R. secalis* encodes a necorsis inducing peptide which functions as a toxin (Rohe et al. 1995). The predicted AvrLm1 protein encoded by *AvrLm1* of *Leptosphaeria maculans* infecting oilseeds rape (*Brassica napus*), is composed of 205 amino acids with a single cysteine residue. It contains a peptide signal suggesting extracellular localization. *AvrLm1* is constitutively expressed, with possible enhanced expression in response to infection by *L. maculans* (Gout et al. 2006).

The interaction between tomato and *Cladosporium fulvum* has been demonstrated to comply with the gene-for-gene system in which recognition of effectors by plant genotypes with matching *R* genes leads to resistance to this pathogen. The *avr9* gene of *C. fulvum* produced a cysteine-rich 28-amino acid peptide which elicited a response in tomato cultivars carrying the *Cf*-9 resistance gene (Kooman-Gersmann et al. 1997). Likewise, the *R* gene *Cf4* from tomato conferred resistance to strains of *C. fulvum* that express *Avr4. Cf* genes encode type I membrane glycoproteins carrying extra cytoplasmic Leu-rich repeats (LRRs) (Thomas et al. 1998). The Cf-4 and Cf-9 proteins possess identical C-terminal regions with sequence variation confined to their N-terminal LRRs. Direct interaction between Cf-9 and Avr9 was not revealed by extensive binding experiments (Luderer et al. 2001). However, AVR 2 from *C. fulvum* has been demonstrated to bind to and inhibit the tomato extracellular cysteine protease RCR 3. This molecular interaction was detected by the extracellular domain of R protein Cf-2 leading to an HR (Rooney et al. 2005).

Signaling components acting downstream of *R* genes have been identified in tomato. *Rcr-1* and *Rcr-2* were required for the function of *Cf-2* but not for other identified *Cf*-genes (Dixon et al. 2000). *Rcr-3* encodes secreted Cys protease and likely functions upstream of *Cf-2* (Krüger et al. 2002). In a later study, additional components required for Cf-mediated defense responses were identified. A collection of Avr9/Cf-9 that rapidly elicited (*ACRE*) genes from tobacco was identified. Many of the *ACRE* genes were shown to encode putative signaling components indicating possible pivotal roles in the initial development of the defense response. By using virus-induced gene silencing (VIGS) approach, three of the 42 genes were found to compromise the Cf-mediated HR in *N. benthamiana*. One of these three genes encoded a Ser/Thr protein kinase called Avr9/Cf-9 induced kinase 1 (ACIK1) which was required for Cf-9/Avr9 and Cf-4-mediated HRs. It was not, however, required for the HR or resistance mediated by other resistance / Avr systems (Rowland et al. 2005).

The classical receptor-elicitor model proposes a direct interaction between the R protein and the corresponding Avr protein. Despite several studies with numerous sets of R and Avr proteins, only two direct interactions had been demonstrated (Tang et al. 1996; Jia et al. 2000). Hence, an alternative model 'guard hypothesis' was proposed by Van der Biezen and Jones (1998). According to this hypothesis, R proteins activate resistance, when they interact with another plant protein (a guardee) that is targeted and modified by the pathogen during invasion to create a favourable environment. The attempt of the pathogen to attack the guardee is detected by R protein, after which resistance is triggered without involvement of R and Avr proteins. In support of the guard hypothesis, an *Arabidopsis* R protein has been detected (Mackey et al. 2002).

Ubiquitinization modulates environmental and endogenous signals in plants, including responses to infection by microbial pathogens. The identity of ubiquitin ligase (E3) involved in regulation of defense by the ubiquitin pathway is not clear. The two *Avr9*/*Cf-9* rapidly elicited (*ACRE)* genes essential for *Cf-9* and *Cf-4* dependent HR have been earlier shown to encode E3 ligases (Rowland et al. 2005). In a further study, *ACRE* genes encoding putative ubiquitin ligases were analysed. *ACRE74* encodes a U-Box E4 ligase homolog, highly related to parsley and *A. thaliana* protein was designated *Nt CMPG1*. Transcript levels of *Nt CMPG1* and the homologous tomato *Cmpg1* were induced in *Cf-9* tobacco and *Cf*-9 tomato after Avr9 elicitation. Tobacco CMPG1 exhibited E3 ligase activity in vitro. *N. benthaminana* plants silenced for *Nt CMPG1* showed reduced HR following elicitation by Cf-9/Avr9. In contrast, over expression of *Nt CMPG1* induced a stronger HR in *Cf-9* tobacco plants, when Avr9 was HRinfiltrated. Silencing of *Cmpg1* decreased resistance to *Cladosporium fulvum* in tomato. The results demonstrated the requirement of the E3 ligase Nt CMPG1 for plant defense and disease resistance (González-Lamothe et al. 2006).

Posttranscriptional gene silencing (PTGS)-based approach aims to reduce the level of expression of a gene of interest. The mechanism of PTGS involves the sequence-specific degradation of RNA. One of the several techniques applied to harness PTGS phenomena is the virus-induced gene silencing (VIGS) approach (Baulcombe 1999; Dinesh-Kumar et al. 2003). The VIGs is a plant RNA-silencing

technique that employs viral vectors carrying a fragment of a desired gene to generate ds RNA which initiates the silencing of the target RNA. Viral genomes have been modified to produce VIGS vectors. The most widely used VIGS vectors are based on *Tobacco rattle virus* (TRV) which have been used to silence genes in many solanceous plants such as tomato, pepper and potato (Liu et al. 2002; Chung et al. 2004; Brigneti et al. 2004). The ability of TRV to infect host meristem tissue is considered as a distinct advantage of using TRV for VIGS. The effectiveness of using TRV VIGS for silencing *Arabidopsis* disease resistance (*R*) genes was investigated. *Arabidopsis* Co1-0 plants carrying *RPM1*gene reacted with HR on inoculation with *P. syringae* carrying *Avr Rmp1* or *AvrB*. TRV VIGS was used to silence *RPM1* in *Arabdiopsis* plants which on inoculation failed to produce HR PCD. But non-silenced plants showed HR PCD. The silenced plants had 92% lower levels of transcripts of *RPM1* gene. The results demonstrated that TRV VIGS is an effective technique to silence *R* genes and can be used as a tool for investigating plant innate immunity (Burch-Smith et al. 2006).

Expression profiling of tomato plants mounting a synchronized HR was carried out to identify genes required for the HR, in addition to functional analysis of differentially expressed genes. The expression profile of tomato plants containing both *Cf-4* resistance gene against *Caldosporium fulvum* and the matching *Avr4* avirulence gene of the pathogens was compared with that of control plants, using cDNA AFLP analysis. The transcript derived fragments (192) were identified as Avr4-responsive tomato (*ART*) fragments, based on their sequence and differential expression. These *ART* fragments were selected for VIGS in *Cf-4* – transgenic *N. benthamiana* plants. The inoculated plants were analyzed for compromised HR by agroinfiltration of either the *C. fulvum Avr4* gene or *inf1* gene of *Phytophthora infestans* which are known to induce HR in *N. benthamiana.* VIGS with fragments of *ART* genes encoding HSP90, a nuclear GTPase, an L19 ribosomal protein and most importantly, a NB-LRR-type protein drastically suppressed HR induced by Avr4 and Inf1. The NB-LRR protein (designated NRC1, for NB-LRR protein required for HR-associated cell death 1) for Cf resistance protein function as well as Inf1-mediated HR was shown to be essential. This finding suggested that signaling pathways may converge and NB-LRR proteins may play a role in signal transduction cascades downstream of resistance proteins (Gabriels et al. 2006).

Whereas the R proteins have a conserved structure, Avr proteins are extremely varied, reflecting the diverse array of pathogenic molecules to which host plants are exposed and which could be used as recognition targets. Colocalization of R and Avr products is presumably required for recognition. In the case biotrophic fungal pathogens such as rusts, powdery mildew and downy mildews specialized structures, haustoria that penetrate the plant cell wall are formed. However, they remain separated from the host cytoplasm by the host cell membrane. Formation of haustoria in resistant plants appears to be the trigger for induction of HR suggesting that rust Avr products are detected at these sites. In flax, (*Linum usitatissimum*), at least 30 rust resistance specificities have been differentiated based on their ability to recognize different strains of rust pathogen *Melampsora lini*. R proteins encoded by genes at the *L*, *M*, *N* and *P* loci are members of the intracellular NBS-LRR class

(Dodds et al. 2000). Although *Avr* genes have been described at genetic level in rusts and mildews, no Avr products have been identified in these pathogens (Zambino et al. 2000; Pedersen et al. 2002).

In a later study, 11 different *L* resistance specificities were identified and the corresponding avirulence genes in *M. lini* were found to map to eight independent loci. An *M. lini* cDNA marker that cosegregated in an F2 rust family with a complex locus determining avirulence on the L5, L6 and L7 resistance genes. Two related avirulence gene candidates, AvrL 567-A and AvrL 567-B were identified in a genomic contig from avirulence allele, while the corresponding virulence allele contained a single copy of a related gene AvrL 567-C. The transcripts of AvrL 567 were detected in RNA samples derived from leaves of flax infected by rust strain C9H5, but not in RNA from germtubes of rust spores germinated in vitro, as revealed by RNA gel blot analysis. Further, AvrL567 transcripts could be detected in RNA from leaves infected with rust strains homozygous for either the virulence or avirulence alleles indicating that the AvrL 567-C gene is expressed in the rust (Fig. 3.2). In confirmation, RT-PCR assay also did not detect any AvrL567 transcript in RNA from rust spores germinated in vitro, while the presence of this transcript could be detected in leaves at 24 h after inoculation. In addition, expression of the rust tubulin and *Sec14* genes also was revealed by RT-PCR at 24 h after inoculation. The purified haustoria contained high concentration of AvrL 567 transcript. The haustorial RNA contained

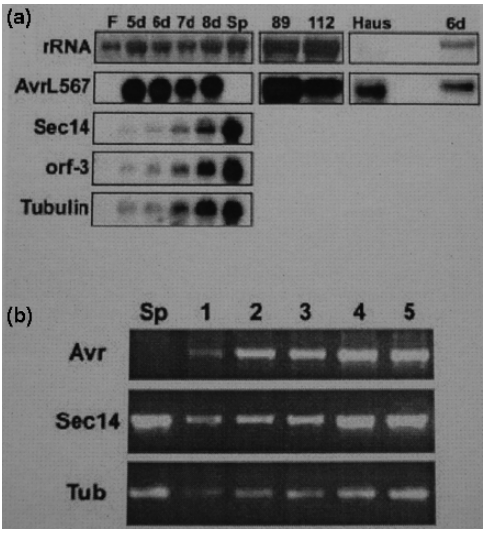

Fig. 3.2 Expression of AvrL567 transcripts in the haustoria of *Melampsora lini* AvrL567 avirulence genes in healthy (F) and rust CH5-infected linseed leaves
(**a**) Probes used for hybridization-*Sec 14, orf 3* and tubulin genes. Note the increase in proportion of rust RNA as the pathogen biomass increased during infection. (b) RNA from germinated rust spore (Sp) and infected linseed leaves after 1 to 5 days subjected to RT-PCR procedure using primers specific for AvrL567, *Sec 14* or tubulin (*Tub*) gene. (Courtesy of Dodds et al. 2004; The American Society of Plant Biologists, Rockville, MD, USA)

4, 2 and 13 cDNA clones derived from AvrL 567-A, AvrL 567-B and AvrL 567-C respectively, indicating that each of the three genes was expressed in haustoria. The AvrL 567 proteins may represent a class of rust effector proteins that are translocated into plant cells to facilitate infection during compatible infection. The existence of any specific translocation mechanism to direct effector proteins into host cells has yet been discovered. The recognition of interaction between the L6 and Avr567 was detected in tobacco resulting in HR induction. This indicates that L6 protein can interact with tobacco resistance-signaling pathway (Dodds et al. 2004).

Recognition by direct R-Avr protein interaction may enhance the possibility of a gene-specific arm race leading to diversification of both R and Avr genes, because such an R protein in the host may be countered by alterations to the pathogen Avr proteins that abolish recognition without loss of effector function and with little or no fitness penalty to the pathogen. The products of *AvrL567* genes of *M. lini* were found to be highly diverse, with 12 sequence variants identified from six pathogen strains. Seven AvrL567 variants derived from Avr alleles induced necrotic responses when expressed in flax plants containing corresponding *R* genes. Yeast two-hybrid assays indicated that there was direct R-Avr protein interaction and this interaction specificity was evident in planta. AvrL567 proteins obtained by cloning in *E. coli* were examined biochemically. The variants that escape recognition may maintain a conserved structure and stability. However, the amino acid sequence differences may directly affect the R-Avr protein interaction. Thus, AvrL567 diversity seems to be the result of R-gene imposed selection in a gene-for-gene arms race. It is important to note that all virulent *M. lini* strains retain and express intact copies of the *AvrL* 567 gene, suggesting a positive fitness value of these genes to this pathogen (Dodds et al. 2006).

In the haustoria of *M. lini*, AvrL 567 proteins are expressed and they contain predicted signal peptides, suggesting that they were secreted into extrahaustorial matrix. However, expression of these proteins in the plant cytoplasm induces an HR, dependent on the cytoplasmic NBS-LRR L5, L6 or L7 resistance proteins of flax. All the flax *R* genes (19) identified encode predicted cytoplasmic TIR-NBS-LRR proteins suggesting that the corresponding rust *Avr* genes encode secreted proteins that could gain entry into the host plant cytoplasm. Hence, haustorially expressed secreted proteins (HESPs) were examined by screening a flax rust haustorium-specific cDNA library for putative secreted peptides. Among 429 unigenes, 21 HESPs were identified, one corresponding to the AvrL 567 gene. Three other HESPs cosegregated with the independent AvrM, AvrP4, and AvrP123 loci. Expression of these genes in flax induced resistance gene-mediated cell death with the appropriate specificity, confirming their avirulence activity. AvrP4 and AvrM induce cell death when expressed intracellularly, suggesting their translocation into plant cells during infection. However, secreted AvrM and AvrP4 could also induce necrotic responses, with secreted AvrP4 more active than intracellular AvrP4. This may be due to enhanced formation of endoplasmic reticulum dependent disulfide bonds. This study indicates that the approach of screening haustorium expressed sequence tags (ESTs) for these encoding predicted signal peptides is an effective method to isolate *Avr* genes from flax rust (Catanzariti et al. 2006).

Resistance to many pathotypes of *Puccinia graminis* f.sp. *tritici* (*Pgt*) causing stem rust disease is governed by *Rpg1 w*hich has also protected barley for over 60 years. *Rpg1* encodes a constitutively expressed protein with two tandem kinase domains. The Rpg1 protein was mainly located in the cytosol and its presence in the plasma membrane and intracellular membranes was also detected. Recombinant Rpg1 autophosphorylated in vitro intramolecularly only serine and threonine amino acids. The site-directed mutagenesis of the two adjacent lysine residues in the ATP anchor of the two kinase domains revealed that the first of the two tandem kinase domains was nonfunctional. Further, lysine 461 of the second domain was shown to be the catalytically active residue. Transgenic barley expressing *Rpg1* mutated in either the kinase 1 or 2 domains were highly susceptible to *Pgt.* The results indicated that both kinase domains were required for resistance to *Pgt.* Furthermore, in planta expressed Rpg1 mutant protein showed that mutation in domain 2, but not 1, rendered the protein incapable of autophosphorylation (Nirmala et al. 2006).

The rice-*Magnaporthe grisea* pathosystem is considered as an attractive model for studying the interaction between an avirulent fungal pathogen and its resistant host plant. Several AVR genes have been identified in this pathogen through classical genetics (Notteghen et al. 1994). The AVR gene from *M. grisea* designated as Avirulence Conferring Enzyme 1 (ACE1) was identified in a cross between Guy 11, hermophrodite isolate from South America and ML25, a male isolate from Africa (Silué et al. 1992b). The rice resistance gene Pi33 was used to recognize the *M. grisea* isolates carrying ACE1 (Berruyer et al. 2003). ACE1 encodes a putative between a polyketide synthase (PKS) and a nonribosomal peptide synthase (NRPS).

The expression of ACE1 during infection was studied by fusing the GFP reporter gene to ACE1 promoter and terminator sequences. GFP fluorescence was not displayed in spore and germtubes of *M. grisea.* GFP fluorescence was first detected in mature appressoria at 15 h after inoculation (hai) of barley or rice leaves and strongest fluorescence was recorded during penetration of the leaf surface ∼24 hai. Disappearance of GFP fluorescence rapidly occurred as the secondary infection hyphae began to spread within leaf tissues. ACE1 was expressed intensively during fungal penetration of host leaves, the point of time when plant defense reactions are triggered. ACE1 seems to be localized in the cytoplasm of the appressorium. The fungal transformants with the vector pACE1 : GFP expressing strong GFP fluorescence in the appressoria were avirulent towards rice cvs. Bala and C101 Lac carrying *Pi33,* while they were fully virulent towards susceptible cv. Maratelli. The results revealed that the ACE 1–GFP fusion protein was functional, since it confirmed avirulence when introduced in a virulent strain of M. grisea. Further, the results suggest that Ace1 enzyme activity is required for avirulence and it is involved in the synthesis of a metabolite that triggers the recognition of the avirulent fungus by rice plants that carry the resistance gene *Pi33.* The fungal signal recognized by resistant rice plants seems to be the secondary metabolite whose synthesis is governed by Ace1 (Böhnert et al. 2004).

The studies on interaction between barley and powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (Bgh) have indicated that the genes *mla* and *mlo* control

different aspects of disease resistance (Jørgensen 1992). The resistance alleles at the *mla* locus are involved in race-specific resistance that is dependent on *Rax1* and *Rar2*. On the other hand, Mlo is responsible for broad-spectrum non-race specific resistance to all powdery mildew races tested and is dependent on *Rar1* and *Rar2* (Jørgensen 1994; Peterhansel et al. 1997). Specific recognition in barley-Bgh interactions is triggered by *Ml* genes in a gene-for-gene manner (Wise 2000). About 30 distinct disease resistance specificities have been recognized at the *Mla* locus on chromosome 5 (IH). Homologues of the down-stream *RAR1* have been located in several plants including *Arabidopsis,* tobacco, rice and maize (Anderson et al. 2005). The *RAR1* protein from barley is composed of two 60 amino acid cysteine- and histidine-rich domains (CHORD) (Shirasu et al. 1999; Halterman and Wise 2004).

The molecular mechanisms of gene-specific barley–*Bgh* interaction were analyzed. The expression of 22,792 barley genes in parallel over the course of *Bgh* infection was evaluated. Twenty two of the barley genes exhibited nearly identical expression patterns among all incompatible interactions up to 16 hai coinciding with conidial germination and appressorial germtube growth. In contrast, divergent expression was noted from 16 to 32 hai during attempted penetration of host epidermal cells and *Bgh* haustorial formation with distinct suppression of most transcripts identified as differentially expressed in compatible interactions. Active suppression of non-specific defense responses was revealed by the coordinated reduction in mRNA abundance of most differentially expressed in compatible interactions. On the other hand, in incompatible interactions, expression of the identified genes generally increased or remained steady from 16 to 32 hai, suggesting that specific recognition of a cognate avirulence effector by the host may lead to maintenance of increased levels of defense-related transcriptors. In order to sustain defense transcript accumulation, there is a need for the host to evolve a mechanism to counter the pathogen's suppression of basal defence or their cellular effects in a cultivar-specific manner (Caldo et al. 2004).

The comparison of *Rar1*-dependent and -independent incompatible interactions provides another layer of defense regulation. The separation of mRNA expression patterns of the selected genes was dependent on whether or not the *Mla* allele required *Rar1* to offer resistance. In other pathosystems, proteinase inhibitors exhibited antifungal activity by inhibiting the pathogen growth (Heath 2000; Shen et al. 2003). Another gene associated with *Rar1*-dependent barley-*Bgh* interaction encodes a predicted Ras-related GTP binding protein, and overexpression of this class of gene in tobacco resulted in formation of abnormally high level of salicylic acid (SA) associated with increase in acidic pathogenesis-related (PR) proteins conferring resistance to *Tobacco mosaic virus* (TMV) (Sano et al. 1994). In contrast, genes associated with Rar1-independent interactions revealed sequence similarity predicted receptor-like kinase and histone H2B-2. Ubiquitinization of receptor kinases commonly associated with eukaryotic cells for protein degradation process does not require RAR1 in *Mla1-AvrMla1* interactions (Caldo et al. 2004). The STG1 protein is involved in the cell cycle regulation in yeast (Azevedo et al. 2002). Silencing of HvSGT1 or HvRAR1 (homologs in barley) in barley epidermal cells resulted

in the reduction in Mla6-triggered resistance confirming that HvSGT1 was involved in HvRAR1-mediated resistance responses. The interaction between HvRAR1 and HSGT1 was confirmed by immunoprecepitation experiments (Azevedo et al. 2002).

The family of plant-specific integral membrane proteins with seven membrane spanning domains is encoded by *HvMlo* in barley and *AtMLO2* in Arabidopsis (Devoto et al. 2003). HvMLO and AtMLO2 proteins are potentially targeted for pathogenesis by powdery mildew pathogens. Barley *mlo* resistance is due to failure of *Blumeria graminis* f.sp. *hordei* (*Bgh*) sporelings to successfully penetrate epidermal host cells. HvMLo was reported to interact with Ca^{2+} sensor calmodulin (Kim et al. 2002) and possibly to inhibit vesicle-associated and SNARE protein-dependent defense reactions (Schulze-Lefert 2004). Following invasion by fungal pathogens, plant cells respond by rapid cellular rearrangements and molecular reprogramming, resulting in host cell polarization toward the intruding fungal pathogens (Lipka and Panstruga 2005). Cell polarization is frequently associated with extensive cytoskeletal rearrangements. The actin cytoskeleton is considered to have a role in establishing barriers at the cell periphery against fungal ingress. The epidermal cells of barley have been demonstrated to require actin cytoskeleton function for basal defense to appropriate pathogen *Bgh* and for *mlo*-meidated resistance at the cell wall, but not for several tested race-specific immune responses. Analysis of nonhost reistance to nonpathogens *Erysiphe pisi* and *E. graminis* f.sp. *tritici*, indicated the existence of actin-dependent and actin-independent resistance pathways functioning at the cell periphery. It is possible that these pathways act synergistically and seem to be under negative control by the plasma membrane-resistant MLO protein (Miklis et al. 2007).

The ubiquitin/proteosome pathway has a role in plant-pathogen interaction. Many genes encoding pathway components like E2 or RING E3 ligases are transcriptionally upregulated by pathogen-derived elicitors (Takai et al. 2002). Several barley R protein including Mla require Rar1 (required for Mla-mediated resistance 1) that by itself interacts with SGT1 (suppressor of G-two allele of SKP1), an SCF-E3 activator (Azevedo et al. 2002). Regulation of protein turnover, possibly through the ubiquitin / proteasome pathway appears to be important for effective defense against infection by fungal pathogens. The role of protein (poly) ubiquitination pathways in basal host defense against *Blumeria graminis* f.sp. *hordei* (*Bgh*) and in nonhost resistance against wheat powdery mildew pathogen *B. graminis* f.sp. *tritici* (*Bgt*) was investigated. The protein turnover and performed transient-induced gene silencing (TIGS) of ubiquitin and 26S proteasome subunit encoding genes in epidermal cells were determined. Partial depletion of cellular ubiquitin levels by TIGS caused extreme susceptibility in transformed cells towards the appropriate host pathogen *Bgh.* On the other hand, papilla-based resistance to non-host pathogen *Bgt* and host resistance mediated by the *mlo* gene was not altered. Cells were rescued from TIGS-induced ubiquitin depletion by synthetic genes encoding wild-type or mutant barley mono-ubiquitin proteins. Systematic RNA interference of 40 genes encoding all 17 subunits of the proteosome 19S regulatory particle failed to induce hyper-susceptibility against *Bgh*. The results suggested a role for the Lys-48-linked

polyubiquitination, which is independent from the proteosome pathway, in basal host defence of barley (Dong et al. 2006).

A major defense mechanism, regulated by jasmonic acid (JA) and ethylene (ET) defences is the HR, a special programmed cell death (PCD) reaction. This reaction is likely to eliminate biotrophic pathogens that need living host tissue for their survival. The HR is an important element of *R* gene-mediated resistance. Another type of defense mechanism is exhibited, when the fungal pathogen penetrates plant cell for establishing infection as in the case powdery mildew pathogens. A local apposition (papilla) is formed on the inner side of the cell wall at the site of penetration. Two mutant alleles *pen1-1* and *pen1-2* of the *PENETRATION1* (*PEN1*) gene required for penetration resistance in *Arabiopsis*-nonhost *Blumeria graminis* f.sp. *hordei* (*Bgh*) interaction were identified (Collins et al. 2003). Use of mutant screens in the same pathosystem resulted in the identification of *pen*-mutants with dysfunctional penetration resistance (Ellis 2006). Formation of the cell wall appositions in response to initiation of infection is controlled by the *PEN1* encoding syntaxin SYP 121. A barley ortholog SYP 121 was required for penetration resistance to *Bgh*, a host pathogen (Collins et al. 2003).

Syntaxins are essential proteins of the SNARE* machinery, controlling vesicle traffic and bulky transport of cargo in cells (Bock et al. 2001). The plasma membrane syntaxin 121 is likely to be involved in secretion of components for the pathogen-induced cell wall appositions. Genetic screens for mutations that result in increased penetration of *Bgh* on *Arabidopsis* have identified the PEN1 syntaxin. Upon pathogen attack, PEN1 appeared to be actively recruited to papillae and a 2 h delay in papillae formation in the *pen1-1* mutant. PEN1 seemed to have a basal function in secretion and a specialized defense – related formation being required for the polarized secretion events that give rise to papilla formation (Assaad et al. 2004). The protein SYP121, together with SYP122 was shown to function as a negative regulator of subsequently induced defence pathways. Necrosis and dwarfism seen in the syntaxin double mutant syp 121-1 syp122-1 was due to dramatic increase in the SA level. By introducing the SA-signaling mutations *eds1-2, eds 5-3, sid2-1* and *npr1-1* as well as the *NahG* transgene, the above phenotype could be partially rescued. These triple mutants exhibited an unknown defense to *P. syringae* pv. *tomato* and an enhanced HR-like responses to nonhost and host powdery mildew pathogens *Bgh* and *E. cichoracearum* respectively. The efficient resistance to *E. cichoracearum* was SA-dependent. The JA/ET signaling marker PDF1.2 was highly upregulated in the triple mutants, indicating SYP121 and SYP122 functioned as negative regulators of PCD, SA, JA and ET pathways through a molecular function distinct from that of SYP121 in penetration resistance. It is possible that individual cells may either express penetration resistance or subsequently induced defences preferentially (Zhang et al. 2007).

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* SNAP (Snaptosome-associated protein); SNARE (SNAP receptor) complex (formed with ROR2 from barley required for basal resistance *Bgh*)

Although more than 25 independent *AVR* genes have been identified in *Blumeria graminis* f.sp. *hordei* (*Bgh*) isolates, none of them has been isolated. The *AVR* genes are distributed throughout the *Bgh* genome and a cluster containing AVR_{K1}, AVR_{a10} *and* $AVRa_{22}$ is found to be linked by 1 to 2 centimorgans (cM) (Brown and Jessop 1995; Caffier et al. 1996). Barley *R* genes (> 85) each conferring resistance to specific *Bgh AVR* elicitors, including *Mlk1* and 28 alleles at the *mla* locus on barley chromosome 5 have been identified (Jørgenson 1994). The avirulence genes Avr_{k1} and AVR_{a10} present in *Bgh* have been identified and they belong to a gene family present in *Bgh* and other *formae speciales* of the grass powdery mildew fungi. The effect of transient expression of AVR_{k1} and AVR_{a10} on penetration success could be assessed, because *Bgh* infects single host epidermal cells. When AVR_{k1} was expressed in resistant barley varieties containing *Mlk1*, the GFP reporter expression was drastically reduced. Cell death did not always occur in cells expressing the *Avr* gene and other changes could have prevented the establishment of successful infection. The *AVR* genes AVR_{x1} and AVR_{a10} were recognized when transiently expressed within host cells, which is consistent with the predicted cytoplasmic location of Mla proteins. These *AVR* genes induced cell death and inaccessibility when transiently expressed in *Mla10* and *Mk1* barley varieties respectively (Ridout et al. 2006).

Infection of plants by *Colletotrichum* spp. is characterized by active penetration of plant cell wall by production of appressoria and penetration pegs. Deposition of a melanin layer in appressoria is essential for development of high internal turgor pressure within appressorium (Volume 2, Chapter 2). Three non-pathogenic strains of *C. lindemuthianum* (causing bean anthracnose disease) with defects in each of the appressorial development were generated. The strain H18 was blocked at the differentiation stage (with no appressorium formation); H191 was blocked at appressorium maturation stage (appressoria defective in pigmentation) and H290 was impaired in appressorium function (failure to penetrate the cell wall). Inoculation of bean with wild-type strain UPS9 and mutant strain H290 elicited strong accumulation of PAL3, CHS and PvPR2 transcripts. The early induction of these genes may be linked to the perception by the plant of fungal conidia upon inoculation and/or conidial adhesion and growth of germtubes on leaves. The mutant strains and the wild-type strain could be discriminated based on the defense responses induced by them in bean plants. Appressorium maturation, but not appressorium function, attained in the wild-type UPS9 and H290 strains, was both necessary and sufficient for triggering most defense responses such as early plant defense genes and accumulation of major PR proteins. However, appressoirum function (entry into the first host cell) was required for avirulence-mediated recognition of the fungus. The results suggested that the avirulence factors may be produced during penetration of cells followed by triggering defense response by the compounds secreted by the fungus or fungal cell wall proteins (Veneault-Fourrey et al. 2005).

An array of disease effector proteins are produced by Oomycetes including *Phytophthora* spp. to reprogramme the defense circuitory of host cells to accomplish parasitic colonization. The effectors are delivered into the host apoplast. The apoplastic effectors interact with extracellular targets and surface receptors, whereas cytoplasmic effectors are translocated inside the plant cell presumably through

specialized structures such as haustoria or infection vesicles that invaginate inside living host cells. The oomycetes effectors including the avirulence (AVR) proteins are recognized only by their ability to activate defense responses and innate immunity. *Phytophthora infestans* secretes inhibitors that target defense proteases in the plant apoplast (Tian et al. 2004). The effectors like glucanase inhibitors and cell death elicitors are also able to function in the host apoplast (Kamoun 2003). Four *Avr* genes have been identified in oomycetes: *Avr1b* in soybean pathogen *Phytophthora sojae, ATR13* and *ATR1* ^{NdWsB} in *Hyaloperonospora parasitica* infecting *Arabidopsis thaliana* (Allen et al. 2004; Rehmany et al. 2005) and Avr3a in *P. infestans* infecting potato (Armstrong et al. 2005).

The *Avr* genes were detected by the presence of cognate R proteins in the host cytoplasm, suggesting that oomycetes can deliver effectors into the host cells, possibly through haustoria. A conserved motif (RXLR) within 32 amino acids of the predicted signal peptides was revealed by sequence alignment of the AVR proteins. The RXLR was hypothesized to function as a singal that may mediate trafficking into host cells (Rehmany et al. 2005). *P. infestans, P. sojae* and *P. ramorum* have a conserved RXLR motif as indicated by bioinformatic analyses. Another *P. infestans* RXLR – containing protein carries a functional nuclear localization signal and possibly accumulate in host nuclei during infection (Birch et al. 2006). Evidence has been obtained to show that *R-Avr* gene interactions may mediate several type of partial host resistance as well as non-host resistance (Kamoun 2001; Song et al. 2003). A broad spectrum of isolates of *P. infestans* was recognized by the *RB* (also termed as *Rpi-blb1*) from *Solanum bulbocastanum* and this gene seems to provide durable resistance under field condition to *P. infestans* (Song et al. 2003). Targeting a pathogen 'Achilles heel' may lead to durable resistance, since mutations in the *Avr* gene would result in a severe fitness penalty for the pathogen (Kamoun and Smart 2005).

Elicitins expressed by *Phytophthora infestans* and other *Phytophthora* spp. form a family of structurally related extracellular proteins that induce HR and other biochemical changes associated with defense responses in *Nicotiana* spp., but not in potato and tomato. Most species of *Phytophthora* produce the well-characterized 10-kDa canonical elicitins (Class I) such as INF1 of *P. infestans* infecting potato and tomato. Elicitins from *Phytophthora* spp. encode small secreted proteins ($<$150 amino acids) with even number of cysteine residues that may induce defense responses when infiltrated into plant tissues (van't Slot and Knogge 2002). Many effectors secreted by oomycetes belong to this category of cystein-rich small proteins. By using PCR amplification with degenerate primers and random sequencing of cDNAs a complex set of elicitin-like genes was isolated. Eight elicitin and elicitin-like genes (*inf* genes) were isolated in *P. infestans.* All these genes encode putative extracellular proteins that share the 78 amino acid elicitin domain corresponding to the mature class I elicitins (INF1) (Bateman et al. 2002). The six genes *inf2A, inf2B, inf5, inf6, inf7* and M-25 encode predicted proteins with a C-terminal domain, in addition to the N-terminal domain common to all elicitins (Kamoun et al. 1997). The Class I elicitin have been shown to bind sterols, such as ergosterol and function as sterol carrier proteins (Boissy et al. 1999), suggesting a biological function of

essential importance to *Phytophthora* spp. As these fungal pathogens cannot synthesize sterols and they have to assimilate them from external sources, role of elicitins appears to be significant. Furthermore, the elicitin-like proteins of *P. capsici* with significant similarity to INF5 and INF6, exhibited phospholipase activity which may be required for lip binding or a processing role for different members of elicitin family (Osman et al. 2001). The gene *M-25* with similarity to elicitins was reported to be induced during mating in *P. infestans* (Fabritius et al. 2002).

The gene inf^{2A} and inf^{2B} encoding a distinct class (Class III of elicitin-like proteins) have also been isolated from *P. infestans*. The *inf2*- like elicitin genes appear to occur as a small genus-specific gene family and are conserved in all tested species of *Phytophthora.* The class III elicitins are considered to be cell-surface-anchored polypeptides. The expression profiles of *inf2A* and *inf2B* genes during infection of tomato by *P. infestans* were determined using semi-quantitative RT-PCR assay. The genes *inf1 and inf2B* expression was observed as early as 1 dai, whereas expression of *inf2A* could be detected only 3 dai. Both *inf2* genes were expressed during *P. infestans* colonization of tomato. Induction of HR by INF1 and INF2A in *Nicotiana benthamiana* was found to be dependent on the ubiquitin ligase-associated protein SGT1. The variation in the resistance of *Nicotiana* spp. to *P. infestans* depended on their response to INF elicitins, as revealed by the positive response of tobacco, but not *N. benthamiana* to INF2B. Comparative analysis of elicitor activity of INF1, INF2A and INF2B using *Potato virus X* (PVX) agroinfection and agroinfiltration demonstrated that INF2A and INF2B induced HR-like symptoms on tobacco, like INF1 and other elicitins. However, a significant difference in the specificity of HR induction could be noted for INF2B which could not induce any necrosis on *N. benthamiana,* while INF1 and INF2A could do so (Huitema et al. 2005).

The requirement of the ubiquitin-associated protein SGT1 for induction of HR by INF2A, as in the case of INF1 was examined, since SGT1 has been found to be a central player in *R*-gene-mediated HR signaling in various plants such as barley, *A. thaliana* and *N. benthamiana* (Peart et al. 2002; Shirasu and Schulze-Lefert 2003). HR induction by INF2A in *N. benthamiana* required the ubiquitin ligase associated protein SGT1. In this respect its requirement was similar to INF1. In order to determine the association of the necrotic response elicited by the INF2 proteins with the induction of defense response genes, leaves of transgenic tobacco line carrying the GUS reporter gene driven by the promoter of the pathogenesis-related (PR) genes *Bgl2* (PR2) with *A. tumefaciens* strains carrying pGR 106-INF1, pGR106 INF2A and pGR106-INF2B were wound inoculated. Both pGR 106-INF1 and pGR106-INF2B elicited increased levels of PR1a expression. The results suggest that INF2B also can induce the expression of PR genes *PR1a* and *Bgl2* in tobacco, like INF1. The elicitin INF2A, however, could not induce PR genes in a significant manner (Huitema et al. 2005).

Recognition of elicitins is considered to be one component of resistance of *Nicotiana* spp. to different *Phytophthora* spp. Evidence for this presumption was obtained by using *P. infestans* strains engineered to be deficient in the elicitin INF1 by gene silencing. The deficient strains induced disease lesions on *N. benthamiana*, suggesting that INF1 conditions avirulence to *N. benthamiana* (Kamoun

et al. 1998b). *P. parasitica* var. *nicotianae* causing black shank disease in tobacco produces the elicitin PARA1. A great majority of the isolates of *P. parasitica* var. *nicotianae* do not produce PARA1. The isolates producing the elicitin exhibited down-regulation of *para1* gene expression in planta (Colas et al. 2001). The results of various studies suggest that elicitins are species-specific avirulence factors. However, some INF1-producing isolates of *P. infestans* could colonize *N. benthamiana* plants, even though this host plant species responds to INF1 elicitins. Elicitins may not always function only as avirulence factors, but may function as general elicitors in some pathosystems (Vleeshouwers et al. 2005).

The mitogen-activated protein kinase (MAPK) cascade is one of the known major pathways by which extracellular stimuli are transduced into intracellular responses in eukaryotic cells via phosphorylation and dephosphorylation of signaling proteins (Davis 2000). The MAPK cascades are composed of three sequentially acting kinase components, MAPKK kinase (MAPKKK), MAPKK and MAPK (He et al. 2006a). A tomato MAPKKKα was identified as a positive regulator of cell death associated with both plant immunity and disease (del Pozo et al. 2004). A high-throughput overexpression of *Nicotiana benthamiana* cDNAs identified a gene for a MAPK as a potent inducer of HR-like cell death. The product of this gene, NbMKK1 protein was found to be localized to the nuclei and the N-terminal putative MAPK docking site of NbMKK1 was required for its function as a cell-death inducer. NbMKK1-mediated cell death was compromised in leaves where NbSIPK expression was silenced by virus-induced gene silencing. *Phytophthora infestans* NF1 elicitin-mediated HR was delayed in NBMKK1-silenced plants, indicating that NbMKK1 was involved in this HR pathway. The resistance of *N. benthamiana* to a non-host pathogen *Pseudomonas cichorii* was compromised in NbMKK1-slilenced plants. The results indicated that nonhost resistance including HR cell death may be due to MAPK cascades involving NBMKK1 (Takahashi et al. 2007).

Botrytis cinerea, a necrotrophic pathogen with a wide host range (>200 plant species), induces host cell collapse indicating possible secretion of toxic metabolite(s). In *Arabidopsis thaliana* cells at a considerable distance from the growing hyphae, accumulation of H_2O_2 was observed before cell death (Govrin and Levine 2000). Later, the intercellular fluid (IF) extracted from *A. thaliana* leaves infected by *B. cinerea* was shown to have elicitor activity. Treatment of intact leaves or cell cultures with either IF from infected leaves or medium from inoculated *A. thaliana* cell cultures induced generation of reactive oxygen species (ROS), resulting in reduction of photosynthesis and electrolyte leakage. Necrotic lesions resembling HR were produced on treated leaves. Necrosis was absent in *dnd1* mutants of *A. thaliana* compromised in HR. *B. cinerea* elicitor appeared to activate the same cell death pathway as the pathogen itself. Analysis of gene expression after treatment with cell-free intercellular fluid from infected leaves revealed induction of several defense pathways including induction of the PR-1 and PR-5 genes and the general stress inducible *GST-1* gene. In addition, IF induced expression of a senescence associated SAG-13 gene (Govrin et al. 2006).

Plant cuticle, in addition to its role as a barrier, may be a source of signals used by invading pathogens to prepare and adjust for the colonization of host tissues. Con-

comitantly plants have the potential to recognize breakdown products of the cuticle and activate defense-related mechanisms. Plants with cuticular defects resulting from overexpression of a fungal cutinase displayed total immunity against *Botrytis cinerea.* The reactions of *A. thaliana* plants that express a fungal cutinase targeted to their cell walls (CUTE plants) or of a mutant body guard (*bdg*) with defects in cuticle structure and integrity were studied. The hyphal growth of *B. cinerea*, but not spore germination was arrested on CUTE plants at an early stage of infection. *B. cinerea* expressed several pathogenicity genes like cuitnase, indicating recognition of the surface of CUTE plants. But neither cellular damage nor tissue colonization by the pathogen was discernible. An inhibitory activity diffusing from leaf surface functioning in vivo was detected. There appeared to be some relationship between the inhibitory effect of the fungitoxic diffusate and development of resistance of the CUTE plants. This fungitoxic diffusate could be the first defense layer against invasion by *B. cinerea* in plants with defective cuticles. The plant defensin, a marker associated with the JA and ET signaling pathways was not induced, indicating resistance of CUTE plants was not related to the induction of PR protein genes. The lack of correlation was consistent with SA-, JA- and ET-independent resistance to *B. cinerea* (Chassot et al. 2007).

Conspicuous priming of the genes belonging *LTP* (lipid transfer protein), *PER* (peroxidase) and *PI* (protein inhibitor) families was recognized in inoculated CUTE and *bodyguard (bdg)* mutant plants by using genome-wide expression analysis. After mock inoculation of *CUTE* and *bdg* plants, marked increase in the expression of the selected genes was observed and this increase was further enhanced following inoculation with *B. cinerea.* The role of LTPs in the formation of cutin and suberin layers and inhibition of growth of fungal pathogens was investigated (Blein et al. 2002). Proteins encoded by class III *PER* gene family in cell wall lignification and cross linking, H_2O_2 generation or detoxification (Tognolli et al. 2002) were studied. Overexpression of proteinase inhibitiors (PIs) from *Nicotiana alata* was shown to protect tobacco plants against *B. cinerea* (Charity et al. 2005). It is possible that action of product of *LTP, PER* and *PI* genes singly or in combination may contribute to the resistance of cuticle-defective plants by providing a several protective shield against the invasion of *B. cinerea*. The results of this investigation revealed that a plant species normally susceptible to the necrotrophic fungal pathogen can show immunity due to cuticular defects and highlight a mechanism that may be exploited to protect plants against the destructive pathogens like *B. cinerea* (Chassot et al. 2007).

The presence of a novel class of necrosis-inducing proteins designated Nep1-like proteins (NLPs) in microorganisms has been detected. This name is derived from the canonical 24-kDa necrosis and ethylene-inducing protein (Nep1) isolated from the purified culture filtrates of *F. oxysporum* f.sp. *erythroxyli* (Bailey et al. 1997; Pemberton and Salmond 2004). NLPs have been shown to induce defense responses in both susceptible and resistant plants. The *PsojNIP* gene in *Phytophthora sojae* was expressed late during the colonization of host soybean tissue during the necrotrophic phase of infection (Qutob et al. 2002). Three cDNAs from *P. infestans* (PiNPPP1.1, PiNPP1.2 and PiNPP1.3) with significant similarity to NLP

family proteins were identified. One of these PiPP1.1 induced necrosis in tomato and *N. benthamiana*, as shown by agroinfection with a binary PVX vector procedure. Expression analyses indicated that PiNPP1.1 was up-regulated during late stages of infection of tomato by *P. infestans.* The necrosis inducing activity of *PiNPP1.1* was compared with INF1 elicitin by employing virus-induced genes silencing (VIGS) technique. The cell death induced by PiNPP1.1 was dependent on the ubiquitin ligase-associated protein SGT1 and the heat-shock protein HsP90. Furthermore, PiNPP1.1 depended for triggering cell death on the defense-signaling proteins COI1, MEK2, NPR1 and TGA2.2, whereas INF1 was not dependent on these proteins for its activity suggesting the requirement of different signaling pathways. Enhancement of cell death was observed due to combined expression of PiNPP1.1 and INF1 in *N. benthamiana*, indicating a possible synergistic interplay between the two cell death responses (Kanneganti et al. 2006).

The other elicitins secreted by pathogenic oomycetes include PcF which is a secreted 52-amino acid peptide of *P. cactorum.* PcF has been shown to trigger necrosis in tomato and strawberry (Orsomando et al. 2001). Elicitation of phenylalanine ammonia lyase (PAL), a defense associated enzyme in tomato was observed following recombinant expression of PcF (Orsomando et al. 2003). GP42, a glycoprotein present abundantly in the cell wall of *Phytophthora sojae* triggered defense gene expression and synthesis of antimicrobial phytoalexins in parsley through binding to a plasma membrane receptor (Nurnberger et al. 1994; Sacks et al. 1995). A cellulose-binding elicitor and lectin-like (CBEL) protein is a 34-kDa cell wall protein first isolated from *Phytophthora parasitica* var *nicotianae*. Elicitation of necrosis and defense gene expression in tobacco and assisting the attachment of cellulosic substrates such as plant surfaces are the assigned dual functions of CBEL (Sejalon-Delmas et al. 1997; Villalba-Mateos et al. 1997).

CBEL contains two cellulose-binding domains (CBDs) belonging to the carbohydrate Binding Module 1 family which is present exclusively in fungi. The necrosis inducing activity of CBEL depends on ethylene and jasmonic acid (Khatib et al. 2004). CBEL was shown to be involved in organized polysaccharide deposition in cell wall and in adhesion of the mycelium to cellulosic substrates (Gaulin et al. 2002). By using modified versions of CBEL protein produced by *E. coli* or synthesized in planta through PVX expression system, the role of CBDs in its eliciting activity was investigated. The recombinant CBEL produced in *E. coli* elicited necrotic lesions and defense gene expression when injected into leaves of tobacco. Likewise, CBEL production in planta induced necrosis. Localization of CBEL in leaf tissues developing small necrosis was monitored by immunogold labeling technique, using the purified CBEL antibody. The protein deposition occurred in the cell wall and junctions of pGR 10: CBEL – infected parenchyma cells. The construct pGR 10: CBEL was based on the entire CBEL coding sequence which was amplified by PCR using CBEL cDNA as a template (Fig. 3.3). The leaf infiltration experiments using synthetic peptides showed that the CBDs of CBEL were essentially required to stimulate defense responses. The pathogen-associated molecular patterns (PAMPs) are recognized by the plants through the receptors and discriminate the ‘self’ and ‘nonself’. CBEL appears to be a PAMP containing molecule from pathogenic fungi

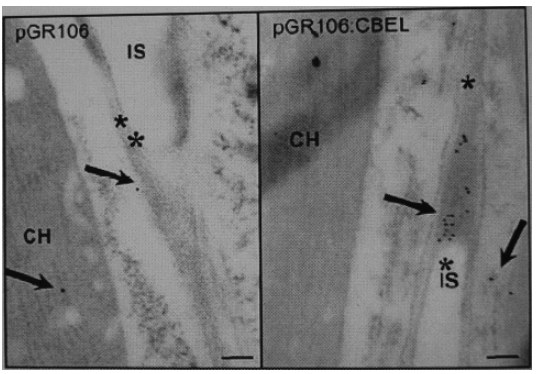

Fig. 3.3 Immunolocalization of cellulose binding elicitor lectin (CBEL) in leaf tissues developing small necrosis at 11 dai with pGR 106 strain of *Phytophthora parasitica* var *nicotianae* using purified polyclonal antibody against CBEL and gold-conjugated goat antiserum to rabbit IgG. *Arrows* indicate gold particles located in the cell wall; asterisks indicate the plant cell wall. CH: Chloroplats; IS: Intercellular space. (Courtesy of Gaulin et al. 2006; The American Society of Plant Biologists, Rockeville, MD, USA)

for which a functional investigation was taken up by generating CBEL-silenced *Phytophthora* mutants. The results showed that CBEL was involved in exogenous cellulose perception and *Phytophthora* cell wall organization suggesting that CBDs of *Ppn* may be considered as PAMPs (Gaulin et al. 2006).

Plant proteases have been shown to have multiple roles in plant defense against microbial pathogens as reflected in their involvement in HR. Proteasome complexes involved in the ubiquitin-mediated protein degradation pathway was implicated in PCD and disease resistance (Tor et al. 2003). The plant vacuolar processing enzymes (VPEs) were suggested to contribute to resistance to basal defense against pathogens during susceptible interactions (Rojo et al. 2004). Among the many proteases that are up-regulated during infection by pathogens, the PR-protein P69B of tomato, an apoplastic subtilisin-like Ser protease was found to accumulate upon infection by *Phytophthora infestans* as well as by *Pseudomonas syringae* (Zhao et al. 2003; Tian et al. 2004). Coevolution of diverse defense counter defense strategies of host and pathogen has become essential in the arms race for survival. Host plants can use PR proteins β-1,3-glucanases for the dissolution of pathogen cell walls rendering the pathogen more susceptible to other plant defense responses or oligosaccharide elicitors may be released activating plant defenses (Kamoun et al. 1998a). However, *Phytophthora sojae*, as a counter defense activity secretes glucanase inhibitor proteins that effectively suppress the β-1,3-glucanases of soybean (Rose et al. 2002). The oomycete pathogens *P. infestans, P. sojae, P. ramorum, P. brassicae* and *Plasmopara halstedii* have been reported to produce a Kazal-like extracellular Ser protease inhibitors belonging to a diverse family that include 35 members (Tian et al. 2004).

In *P. infestans,* two of the 14 Kazal-like inhibitors, EPI1 and EPI10 bound and inhibited the PRP69B subtilisin-like Ser Protease of tomato (Tian et al 2005a). Inhibition of P69B by two structurally different protease inhibitors of *P. infestans*

suggested that EPI1 and EPI10 could function in counter defense types of proteases such as extracellular aspartic protease (AP) and two Cys proteases, CYP and StCath B were up-regulated during infection of potato by *P. infestans* (Guevara et al. 2002; Avrova et al. 2004). Induction of AP expression occurred faster and reached higher concentration in resistant potato cultivar compared with the susceptible one (Guevara et al. 2002). A new family of secreted proteins EPIC1 to EPIC4 with similarity to cystatin–like protease inhibitor domains. Among these, the *epiC1* and *epiC2* genes present in *P. infestans* lacked orthologs in *P. sojae* and *P. ramorum* were relatively fast evolving within *P. infestans* and were up-regulated during infection of tomato, suggesting a role during *P. infestans*–tomato interactions. Tests to assess biochemical functions indicated that EPIC2B could interact and inhibit by novel papain-like extracellular cysteine protease, termed Phytophthora Inhibited Protease 1 (PIP1). It has been established that PIP1 is a PR protein closely similar to Rcr3, a tomato apoplastic cysteine protease that functions in fungal resistance. PIP1 and Rcr3 may function in different aspects of plant defense including perception of invading microbial pathogens, mediation of defense signaling and execution of defense responses (Tian et al. 2007).

Isoflavones have been reported to function as preformed antibiotics and as precursors for the defense-related coumestan and pterocarpan phytoalexins (Rivera-Vargas et al. 1993). Isoflavone synthase (IFS) is the key enzyme in the biosynthesis of isoflavones and it is encoded in soybean by two genes *IFS-1* and *IFS-2* (Jung et al. 2000). The effect of RNAi silencing of genes for this enzyme on the infection of both root and cotyledon tissues of soybean by *Phytophthora sojae* was assessed. Infection of roots of cv. Williams 82 by race 1 of *P. sojae* normally resulted in a resistance response due to the presence of the *Rps1k* gene for resistance to race 1. Soybean cotyledon tissues were transformed with *Agrobacterium rhizogenes* carrying an RNAi silencing construct designed to silence expression of both copies of IFS genes. Infection of IFS RNAi silenced root tissues progressed without any obstruction because of a near total silencing of isoflavone accumulation leading to abolition of *R* gene – mediated resistance in transformed tissues. Although cv. Harsoy carries the *Rps7* gene, it is susceptible to race 1. IFS silencing enhanced the susceptibility of cv. Harsoy further and to a higher level compared to the susceptibility of silenced cv. Williams 82. Silencing of IFS was demonstrated throughout the entire cotyledon (in tissues distal to the transformation site) by HPLC analysis of isoflavones and by real time PCR. A near complete suppression of mRNA accumulation for both IFS1 and IFS2 gene was observed following distal silencing induced by wounding or treatment with cell wall glucan elicitor from *P. sojae*. Silencing of IFS disrupted both R gene-mediated resistance in roots and non race-specific resistance in cotyledons tissues (Subramanian et al. 2005).

Germin is well known as a marker protein in germinating wheat (*Triticum aestivum*) seed. Germin and all analyzed germin-like proteins (GLPs) possess N-terminal secretary signals, suggesting a role in cell wall function or defense against invading pathogens. This is because of oxalate-oxidase (OXOX) or superperoxidase dismutase (SOD) activities of several GLPs leading to H_2O_2 which is considered as a signal molecule for a range of defense reactions (Christensen et al. 2004). In addition,

many GLP-encoding genes are induced following pathogenic infection as in transgenic peanut (groundnut) plants expressing a barley oxalate oxidase gene, inoculated with *Sclerotinia minor* (Livingstone et al. 2005). Based on extensive sequence information from genome or expressed sequence tag (EST) sequencing studies, germin and GLPs belonging to a multigene family in different plants including barley have been detected. In barley, six GLP subfamilies (HUGER 1 to 6) have been identified. The expression of these GLP subfamilies of barley was studied by selecting single mRNAs for gene expression studies as well as overexpression and gene-silencing experiments in barley and *Arabidopsis thaliana.* Infection by the powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (Bgh) and exogenous H_2O_2 application resulted in the strongest signals for induction of several GLP subfamilies. The transcripts of four GLP subfamilies accumulated predominantly in the leaf epidermis. Transient overexpression of HvGER4 or HvGER5 as well as transient silencing by RNA interference of HvGER3 or HvGER5 provided protection to *Bgh* that normally infects the epidermal cells. In contrast, silencing of the HvGER4 resulted in hypersusceptibility. The results revealed that HvGER5 was a new extracellular superoxide dismutase and protection by overexpression was found to be dependent on SOD activity of the encoded protein (Zimmermann et al. 2006).

3.1.2.2 Plant Defense Mechanisms

Three types of active defense responses, initiated after the recognition of the pathogens, have been differentiated: (i) The primary responses are seen in the cells that remain in contact with the pathogen or infected by the pathogen (as in the case of viruses); the cells recognize the specific signal molecules of pathogen origin; the outcome of this primary response leads to programmed cell death (PCD), resulting in the development of visible necrotic lesions. (ii) The secondary responses are exhibited by the adjacent cells surrounding the initial infection site in response to diffusible signal molecules (elicitors) formed following primary interaction; the elicitors initiate the activation of plant defense response genes in cultivars carrying the matching or complementary disease resistance gene(s); several of these genes have leucine-rich repeat (LRR) domains which define the specificity for elicitor recognition. (iii) The third type of active defense responses constitutes the systemic acquired resistance (SAR) observed in organs (tissues far away from the site of induction of resistance). The SAR is hormonally induced throughout the plant.

Basal Resistance (Local Response)

Various defense mechanisms are induced in plants exposed to microbial pathogens. The extent of activation may vary depending on the levels of resistance to the pathogen species or race concerned. In a non-host plant species, the nonself recognition system perceives at the site of attempted penetration typical microbe (pathogen)-associated molecular patterns (MAMPs/PAMPs). The MAMPs recognized by non-host plant cells are chitin fragments (chitooligosaccharides) released from fungal cell walls during pathogen attack, eliciting strong plant defense responses (Knogge

and Scheel 2006). Deposition of mechanical barriers such as carbohydrates and hydroxyproline-rich glycoproteins within cell walls to restrict the development of fungal hyphae, synthesis of small secondary metabolites such as phytoalexins with fungitoxic properties and production of active oxygen species are the defense mechanisms operating in infected plants. Further, several defense-related enzymes and small peptides with antimicrobial activity such as thionins, defensins and lipid transfer proteins, in addition to accumulation of pathogenesis-related (PR) – proteins have also been shown to the produced in response to infection by fungal pathogens (Narayanasamy 2002, 2006).

The active defense of plants, elicited against invading plant pathogens constitute in a localized manner such as rapid programmed cell death termed as hypersensitive response (HR), the generation of reactive oxygen species (ROS), the activation of a complex array of defense genes and the production of antimicrobial phytoalexins (Dangl and Jones 2001; Torres and Dangl 2005). In addition to these local responses, the distal uninfected plant tissues usually develop systemic acquired resistance (SAR) which is expressed as increased levels of resistance to a subsequent challenge by microbial pathogens. Induction of these defense responses is regulated by complex signaling network initiated after the plant recognition of the pathogens which is mediated either by gene-for-gene interactions between host plant *R* genes and pathogen avirulence (*Avr*) genes or by the binding of nonhost-specific elicitors also termed as pathogen-(microbe) associated molecular patterns (PAMP/MAMP), to their receptors (Dangl and Jones 2001; Ausubel 2005; Boller 2005).

Reactive oxygen species (ROS) is produced within a few minutes after a microbial pathogen is recognized by an incompatible host plant species, probably as the first detectable host response. While ROS can react with other molecules without the input of energy, molecular oxygen (O_2) is relatively unreactive. ROS includes the superoxide anion (O_2^-), H_2O_2 and the hydroxyl radical (OH^-) constituting the oxidative burst and have been established as a characteristic feature of the HR (Bolwell 1999). H_2O_2 (a stable and less reactive ROS) from the oxidative burst plays a central role in the expression of HR. To avoid the adverse effects of oxidation, plants have developed enzymatic systems for scavenging these highly reactive forms of O_2. Superoxidase dismutase (SOD) catalyzes the conversion of O_2^- to O_2 and H_2O_2. Catalase (CAT) and/or ascorbate peroxidase (APX), inturn, convert H_2O_2 into water and O_2. In addition, the extracellular class III peroxidases (POX) catalyze the oxireudction between $H_2O_2$and several other reductants and their activity has been correlated with plant defense against pathogens (Jwa et al. 2006). ROS constituting a likely signal in the cascade leading to cell death during plant-pathogen interactions was suggested by Lamb and Dixon (1997) and Van Camp et al. (1998). Avirulent pathogens successfully recognized via the action of *R* gene products in plant immune system, elicit a biphasic ROS accumulation with a low-amplitude, transient phase, followed by a sustained phase of much higher magnitude that correlates with disease resistance. On the other hand, virulent pathogens may avoid host recognition and induce only the first phase of the response, suggesting a role for ROS in the establishment of host defense. Elicitors of defense responses known as microbe (pathogen)-associated molecular patterns (MAMPs), also may trigger an oxidative

burst affecting the pathogen/or host cells. Alternatively MAMPs may function as signaling molecules that are not directly involved in the mechanism that actually arrest the growth of the pathogens. The regulatory functions for ROS in defense may occur in conjunction with other plant signaling molecules such as salicylic acid (SA) and nitric oxide (NO) (Torres et al. 2006).

The involvement of NADPH oxidase in ROS production during the HR was indicated by Kawasaki et al. (1999). For the production of superoxide from molecular oxygen, the plasma membrane-associated NADPH oxidase may be employed as the manmalia neutrophils for defense. The superoxide anions produced outside the plant cells usually are rapidly converted into H_2O_2, a molecule that can cross the plasma membrane and enter plant cells. Then H_2O_2 is eventually removed from cells by conversion to water through the action of catalase, ascarbate peroxidase or glutathion peroxidase. H_2O_2 may be either directly toxic to the pathogens or able to induce genes for proteins involved in certain cell protection mechanisms such as glutalthione (Hammond-Kosack and Jones 2000). H_2O_2 has been linked to cell wall defenses such as callose deposition, phenolics and protein cross-linking (Borden and Higgin 2002; Mellersh et al. 2002). Callose and oxidatively cross-linked proteins are rapidly produced upon initial pathogen attack and serve to strengthen the cell wall (Bradley et al. 1992; Brisson et al. 1994). Callose composed of 1,3-β-glucan is deposited at appressorial penetration sites forming papillae which may prevent penetration of the cell wall by the fungal pathogen (Aist 1976). Protein cross-linking occurs, when hydroxyproline-rich proteins in the cell wall undergo oxidative cross-linking, a highly regulated response through the mediation by H_2O_2. The insoluble cross-linked proteins strengthen the cell wall and may be useful to trap the pathogen inside of a cell that may show HR (Bradley et al. 1992; Brisson et al. 1994).

In the tomato-*Colletotrichum coccodes* pathosystem, the involvement of H_2O_2 in defense response was indicated. The initial resistance to *C. coccodes* was not found to be associated with active transcription and translation or hypersensitive cell death and removal of H_2O_2 with catalase allowed immediate penetration and development of symptoms of the disease. H_2O_2 accumulated in the area around the appressorium and it was associated with oxidative cross-linking of the plant cell wall proteins (Mellersh et al. 2002). Accumulation of H_2O_2 in response to *Blumeria graminis* f.sp. *tritici* in primary germtube (PGT) and appressorial germtube (AGT) contact and papilla (cell wall apposition) and during hypersensitive responses in wheat (*Triticum aestivum*) was assessed. For this purpose primary leaves of three susceptible wheat lines and five resistant lines were used. Strong H_2O_2 accumulation was noted in effective papillae and associated cytosolic vesicles in both susceptible and resistant wheat lines, suggesting the critical role of H_2O_2 in effective papillae formation as a general plant defense against powdery mildew. All the five resistant lines showed high frequency of effective papillar formation. Hypersensitive cell death in all resistant lines was associated with H_2O_2 accumulation in infected epidermal cells. Penetration resistance but not HR seemed to be mediated by the *Pm2* gene in one of the resistant lines, suggesting that *Pm2* may be responsible for an HR-dependent defense pathway in this genetic background. Papilla deposition is likely to be independent of HR, since both do not occur in the same host cell.

However, both defense mechanisms were found to be associated with high level of H_2O_2 concentration. HR acted as a second line of defense to restrict the invasion of the pathogen, when papilla defense failed in this pathosystem (Li et al. 2005a).

Floral nectar is produced by many plant species and it is a rich source of carbohydrates, aminoacids and other metabolic components. Two proteins Nectarin I and Nectarin V present in the nectar actively generate H_2O_2 via different mechanisms. Nectarin I, a germin-like protein has manganese superoxide dismutase activity (Lou and Baldwin 2006) and nectarin V has Glc oxidase activity. Both are involved in the production of H_2O_2 in the nectar. H_2O_2 produced from the nectar redox cycle was shown to be a major factor contributing to inhibition of most microbial growth in floral nectar. However, some floral pathogens like *Erwinia amylovora* are able to overcome the obstacle of inhibition by H_2O_2. Superoxide production was localized near nectary pores. The mechanism of H_2O_2 production in the nectar was studied. By applying native PAGE assays, the production of superoxide was found to be due to an NADPH oxidase. Studies on temporal expression patterns demonstrated that the superoxidase production (NADPH oxidase activity) was coordinated with nectar secretion, the expression of Nectarin I (a superoxidase dismutase nectar) and the expression of *NOX1*, a putative gene for a nectary NADPH oxidase that was cloned from nectaries and identified as an rboh D-like NADPH oxidase. The NADPH oxidase was expressed in the early stages, implicating posttranslation regulation of the NADPH oxidase in the nectary. Thus H_2O_2 production in floral nectar forms a host defense strategy to ward off most microbial pathogens (Carter et al. 2007).

The ROS production was shown to be associated with formation of defensive barriers against powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). The presence of ROS in the vesicles inside the cells of infected barley plants was observed suggesting that polarized delivery of ROS, among other factors, may lead to inhibition of pathogen development (Huckelhoven and Kogel 2003; Collins et al. 2003). Synergistic action of ROS and salicylic acid (SA), another signal molecules involved in local and systemic defense response for signal amplification loop with SA was proposed for development of HR and subsequent establishment of systemic defenses (Draper 1997). SA accumulation can also downregulate those ROS-scavenging systems. This in turn may contribute to enhanced overall ROS levels following pathogen recognition (Klessig et al. 2000). Calcium metabolism has been demonstrated to be intimately related to ROS signaling. High levels of cytosolic Ca^{2+} are among fastest responses following pathogen infection. Ca^{2+} influx is required for ROS production after elicitation as revealed by experiments using specific inhibitors (Grant et al. 2000). Responses associated with ROS can interact with ethylene signaling. Ethylene induces PCD and senescence. Both ROS and ethylene are implicated in signaling response to *Cauliflower mosaic virus* infection in *Arabidopsis* (de Jong et al. 2002; Love et al. 2005). ROS signaling interacts with many other regulatory events in a complex network of signals that govern the response to pathogens and other factors. This cross talk may be responsible for the wide range of responses mediated by ROS (Torres et al. 2006).

The effect of infection of strawberry by *Mycosphaerella fragariae* on total superoxide dismutase (SOD) activity and induction of SOD isozymes was assessed in three

cultivars which are resistant (Joliette), partially resistant (Honeoye) and susceptible (Kent). The activity changes of SOD were used as an indicator of changes in superoxide production, because of the difficulty in measuring superoxide production in vivo. The antioxidant levels were high in all three cultivars on the second day after inoculation. The resistant and partially resistant plants showed further increase in total SOD concentration, indicating higher tolerance of these cultivars to the oxidative stress induced by *M. fragariae*. The presence of two newly synthesized isozymes of SOD were detected only in the inoculated resistant and partially resistant cultivars, indicating their association with resistance to the leaf spot disease. This approach has the potential for use in breeding programs to select genotypes/cultivars with specific isozyme(s) associated with resistance to the disease(s) (Ehsani-Moghaddam et al. 2006).

The generation of ROS molecules has been a widely accepted as a form of plant resistance to infection by microbial pathogens in a variety of ways. In contrast, in some pathosystems such as oats-*Drechslera avenae*, oxidative forces may function as pathogenesis factors. Both lipid peroxidation and lipoxygenase activity increased in oats infected with *D. avenae* (Von Gönner et al. 1993). Furthermore, cell death caused by *Botrytis cinerea* in bean leaves was associated with ROS generation (Von Tiedemann 1997). Pathotypes of *Alternaria alteranta* infecting Japanese pear produce two different host-specific toxins (HSTs) AK-toxin I and II. The genes *AKT1* and *AKT2* required for toxin biosynthesis have been isolated and the toxin was demonstrated to be an essential factor for expression of pathogenicity. ROS was produced in AKT-toxin I-treated host cells. AK toxin-I induced plasma membrane-derived fragments only in sensitive Japanese pear tissues. Abundant H_2O_2 generation was observed in both the membrane fragments and plasma membranes of toxin-treated sensitive tissues. The results suggested that the membrane modification and lipid peroxidation following ROS generation might have facilitated the successful establishment of infection by *A. alternata* (Shimizu et al. 2006).

Verticillium dahliae causing wilt disease in cotton and other crops produces both low- and high-molecular weight compounds that can function as elicitors and phytotoxins capable of enhancing host defense and faciliting pathogenesis. About 1000 ESTs were generated and many were found to encode proteins harboring putative signal peptides for secretion. Heterologous expression resulted in the identification of a protein designated *V. dahliae* necrosis- and ethylene-inducing protein (Vd NEP) composed of 233 amino acids. Vd NEP acted as a wilt inducing factor on excised cotton leaves and cotyledens. But it induced phytoalexins production and programmed cell death (PCD) of cotton suspension-cultured cells. The bacterially-expressed fusion protein of His-VdNEP also induced necrotic lesions in *N. benthamiana* leaves and a complex of defense responses in *A. thaliana* plants. His-Vd NEP protein induced *PR-1* and *PDF1.2* gene expression in *A. thaliana* plants, in addition to triggering production of ROS. Addition of the fusion protein at low concentration to suspension cultured cotton cells (*Gossypium arboreum*), elicitation of biosynthesis of gossypol and related sesquiterpene phytoalexins was observed. On the other hand, at higher concentrations fusion protein cell death was induced. A low level of *VdNEP* expression in the mycelium of *V. dahliae* in culture was revealed by Northern blotting analysis (Wang et al. 2004a).

Nitric oxide (NO) is an inhibitor of the respiratory cytochrome c oxidase in plant mitochondria and can generate cytotoxic compounds by reacting with ROS. The generation of NO has been shown to enhance H_2O_2–mediated cell death and defense mechanisms (Delledonne et al. 1998). In plants, rapid de novo synthesis of NO accompanies the recognition of avirulent bacteria. NO has the capacity to potentiate induction of plant cell death by ROS. Addition of NO-generating compound to plant cell suspension cultures and leaves results in the accumulation of mRNAs from several genes involved in defense and cell protection. Further, when NO production is inhibited, development of HR diminishes, leading to production of more severe disease symptoms and enhancement of bacterial growth. NO and ROS appear to play an important synergistic role in the rapid activation of a wide repertoire of defense responses after pathogen attack (Hammond-Kosack and Jones 2000).

In compatible host-pathogen interactions involving a HR, NO and ROS such as H_2O_2 and superoxide (O_2^-) may either directly reduce the ability of the pathogen to colonize its hosts or act as signaling molecules by inducing defense-related genes (Hancock et al. 2002). In soybean suspension cells, NO and H_2O_2 synergistically induce hypersensitive cell death (Delledonne et al. 2001). The role of NO in the latent infection of tomato by *Colletotrichum coccodes* was studied using inhibitors of NO. The effects of NO on superoxide (O_2^-) levels on H_2O_2 levels as measured by oxidatively cross-linked proteins and on callose deposition were assessed. Increased levels of O_2^- and reduction in callose depositions and oxidative cross-linking at appressorial sites were noted when NO was reduced. In contrast, increased NO resulted in a greater percentage of appressorial sites with callose and cross-linked proteins. Catalase also, like superoxide dismutase, reduced the amount of protein cross-linking. The results suggest a role for NO in the initial defense of tomato against *C. coccodes* via an effect on plant cell wall modifications at sites of appressoria formation. There appeared to be a balanced relationship of NO with other reactive oxygen species. The effect is possibly to allow sufficient H_2O_2 to facilitate non-lethal cell wall defenses which may temporarily stop colonization by this fungal pathogen (Wang and Higgins 2006). The cell wall associated defenses that function to restrict initial infection are regulated by the balance in quantities of NO, O_2^- and H_2O_2 of which the latter appears to be more important in cross-linking of proteins and other structural polymers in the plant cell wall at the point of potential pathogen penetration. The role of NO in coordination with other activated oxygen species in host defense expression is well coroborated (Delledonne 2005; Wang and Higgins 2006).

Nitric oxide (NO) is considered to function as a key signal in plant disease resistance, since inhibitors of NO accumulation blocked the localized cell death (HR response). The efficient induction of HR is dependent on an appropriate balance between ROS, intermediates (ROIs) and NO production (Delledonne et al. 2001). NO reacts rapidly with glutathione (GSH), the major intracellular low-molecular-mass oxidant to yield S-nitrosoglutathione (GSNO) which may act both as NO reservoir and NO donor (Lindermayr et al. 2005). GSNO reductase (GSNOR) seems to be involved in the regulation of NO system by breakdown of GSNO. The involvement of GSNOR in the basal disease resistance and resistance gene-mediated resistance

against *Peronospora parasitica* and *Pseudomonas syringae* pv. *maulicola* infecting *Arabidopsis.* GSNOR is encoded by single-copy gene in *Arabidopsis*. The transgenic plants with decreased amounts of GSNOR exhibited higher level of basal resistance to *P. parasitica* which correlated with higher levels of intracellular S-nitrosothiols (SNOs) and constitutive activation of *PR-1* gene. In addition, SAR was impaired in plants overexpressing GSNOR and enhanced in the antisense plants. These changes in resistance was related to changes in SNO content both in local and systemic leaves. GSNOR was found to be localized in the phloem and it might regulate SAR signal transport through vascular system. GSNOR controlled SNO in vivo levels which might positively influence both basic resistance and *R*-gene-mediated resistance too. The results bring into focus GSNOR as an important and widely utilized component of resistance protein signaling networks conserved in plants as well as in animals (Rustérucci et al. 2007).

Systemic Host Responses

Plant responses to infection by microbial pathogens, involve induction of expression of a large array of genes encoding diverse proteins having some role in host plant defense. Recognition of the presence of the pathogen by the host plant initiates the process of development of systemic acquired resistance (SAR). Signals are released from the point of infection/penetration by the pathogen triggering resistance in adjacent and also distant tissues. Following inoculation of leaves, certain families of genes collectively known as 'SAR genes' are activated (Ward et al. 1991). The time taken for expression of SAR gene(s) may vary depending on the nature of the biotic or abiotic agent inducing resistance. Plant growth-promoting rhizobacteria (PGPR) applied in soil localize at the surface of roots of treated plants, but they are able to induce systemic resistance (ISR) in leaves and stems for away from the root surfaces where PGPRs remain. SAR and ISR have two phases. All events leading to the establishment of resistance are included in the initiation phase which is transient. During the second maintenance phase, quasi-steady-state resistance occurs as a result of events of the initial phase (Ryals et al. 1994). Infection by a microbial pathogen can shift both proximal and distal plant tissues to a physiological state of elevated defensiveness against a broad range of pathogens. Although SAR and ISR are related, they are distinct versions of this systemic host response. They share two components viz., (i) elevated production of antimicrobial compounds and (ii) potentiation of the defense activation machinery so that antimicrobial responses are activated more strongly and rapidly in response to subsequent infections (Feys and Parker 2000; Dong 2001).

The cellular responses of a plant challenged with a microbial plant pathogen are tightly orchestrated and recent developments have permitted a full analysis of these responses. Two approaches viz., microarray and proteomic analyses have allowed a global analysis of cellular regulation. Microarray based expression profiling methods, together with the availability of genomic and / or EST sequence data for some plant species have enabled characterization of plant pathogenesis-related responses. It has been possible to identify an amazing number of genes associated

with host plant resistance to diseases. By applying DNA microarray technologies, it is possible to monitor the expression of thousands of genes simultaneously, to identify new pathogenesis-related genes, to identify co-regulated genes and the associated regulatory systems and to determine the interactions between different signaling pathways (Harmer and Kay 2001; Kazan et al. 2001). In plants inoculated with *Cochilobolus carbonum*, using a maize DNA microarray respresenting 1500 maize genes, altered mRNA expression of 117 genes consistently at 6 hai was recorded by Baldwin et al. (1999). Using a targeted microarray containing *Arabidopsis* genes (2375), 705 genes that were responsive to *Alternaria brassicola* or to defense-activating signaling molecules salicylic acid (SA), methyl jasmonate (MeJ) or ethylene, were identified (Schenk et al. 2000). Later, jasmonate-responsive genes (41) in *Arabidopsis* and five genes involved in JA biosynthesis and three genes involved in other signaling pathways (possibly in ethylene, auxin and SA) were identified (Sasaki et al. 2001). These studies show that DNA microarrays have the potential for simultaneous identification and hypothesizing functions for many genes that may be involved in plant-defense signaling. It can be expected that many new defense-related genes will be identified in plants as more genomes and ESTs are sequenced and as DNA microarrays become less expensive and more easily accessible to research workers (Wan et al. 2002).

Expressed sequence tags (ESTs) are partial sequences of cDNA clones in an expressed cDNA library. They may be used to identify all of the unique sequences (genes) to determine their functions (Mekhedov et al. 2000). A cDNA microarray can be fabricated using the identified unique cDNA sequences for the functional study. The expression patterns of the resistance genes or cDNAs related to the resistance or susceptibility to the peanut (groundnut) late leaf spot disease were identified and characterized using microarray analysis and real-time PCR assay. The gene expression profiles in peanut genotypes, resistant or susceptible to leaf spot disease, were studied using cDNA microarray containing 384 unigenes selected from two EST cDNA libraries challenged by biotic and abiotic stresses. By comparison of the differential expression of genes of two genotypes, *Cercosporidium personatum* causing late leaf spot disease was found to either stimulate or restrain gene expression in the resistant or susceptible genotypes respectively. In resistant genotype (C34-24), more up-regulated genes were detected, whereas downregulated genes were more numerous in susceptible genotype (GT-Y20). Superoxide dismutase and gluthione-S-transferase 8, the antioxidative proteins were expressed in both genotypes, the expression of the latter enzyme gene being greater in susceptible genotype. The identification of gene whose expression in resistant genotype by microarray analysis, was validated by real-time PCR assay (Luo et al. 2005). The genes encoding peroxidases from a cDNA library from leaf epidermis of diploid wheat (*Triticoccum monococcum*) infected by *B. graminis* f.sp. *tritici* (*Bgt*) were screened. From 2500 expressed sequence tags (ESTs), 36 cDNAs representing 10 peroxidase genes (*TmPRX1* to *TmPRX10*) were isolated and characterized. Four distinct groups of peroxidases were recognized. Differential expression and tissue specific localization among the members were noted during *Bgt* infection by using Northern blots and RT-PCR analysis. Among the peroxidase genes, *TmPRX1*

exhibited highest induction during infection. *TmPRX1* to *TmPRX6* were expressed predominantly in mesophylls, while *TmPRX7* to *TmPRX10* were expressed primarily in epidermal cells. The terminal propeptide was shown to be sufficient to target a GFP protein fusion protein to vacuoles in onion cells (Liu et al. 2005).

The ESTs may form part of an effective ascochyta blight resistance in chickpea. This report appears to the first to indicate the usefulness of cDNA microarrays to study chickpea resistance response to this fungal pathogen (Coram and Pang 2005a,b). In order to identify the ESTs involved in defense responses of *Lathyrus sativus* (grasspea or chickling pea) inoculated with *Mycosphaerella pinodes* causing ascochyta blight, a non-directional cDNA library was constructed from leaf and stem tissue collected from a resistant accession (ATC 80878) at 48 and 72 h post inoculation. Twenty nine unique potential defense related ESTs representing genes coding for enzymes and proteins involved in different levels of defense including the recognition events between plant and pathogen, signaling transduction and regulation, oxidative stress, and HR and specific defense-related pathogenesis-related (PR) proteins. Of the 29 ESTs, 16 grouped in cluster 3 were expressed earlier and/or at greater levels in stem tissue in the resistant genotype than in the susceptible genotype (AC 80407). This investigation has revealed the simplicity and power of using EST approach to assess gene activities during plant-pathogen interaction (Skiba et al. 2005).

Microarrays technology was applied to study the expression patterns of the 20 defense-related ESTs a leucine zipper protein, SNAKIN2 antimicrobial peptide precursor and elicitor, induced receptor protein in a highly resistant chickpea (*Cicer arietinum*) accession (ICC 3996) and a susceptible cultivar Lasseter after inoculation with spores of *Ascochyta rabiei* causing the ascochyta blight disease. The use of a time-series helped the putative detection of gene induction over the sampled period. Three defense-related ESTs exhibited differential up-regulation in the resistant accession when compared to the susceptible cultivar. The chickpea accession ICC3996 showed resistance to the disease but it had agronomically poor traits. This genotype was used to generate an enriched library of EST sequences. The ESTs (1021) were clustered and assembled into 516 unigenes of which 4% were defense-related, encoding lignin and phytoalexin biosynthesis genes, PR-proteins, signaling proteins and putative defensive proteins (Coram and Pang 2005b). In the further study, the resistance responses of four chickpea genotypes, resistant ICC 3996 (IC), moderately resistant FLIP94-508C (FL) and ILWC 245 (IL, *Cicer echinospermum*) and susceptible, Lasseter (LA) were investigated using microarray technology and a set of unigenes of chickpea, grasspea (*Lathyrus sativus*) ESTs and lentil (*Lens culinaris*) resistance gene analogs (RGAs). Microarray observations were validated by quantitative RT-PCR assay. The time course expression patterns of 756 microarray features led to the differential expression of 97 genes in at least one genotype at one time point. The transcriptional changes recorded in the early stages of infection (6–12 hpi) may reflect initial responses following the recognition of pathogen contact and the major responses at 24–48 hpi. may be related to pathogen penetration and signaling cascades that result in an oxidative burst, induction of HR and synthesis of antifungal proteins (Coram and Pang 2006).

The transcription of several putative PR-proteins was significantly induced in resistant genotypes at an earlier time point than in susceptible LA genotype. The specific microarray expression profile of the PR protein β-1,3-glucanase revealed exclusive up-regulation in the resistant genotype IC. Quantitative RT-PCR confirmed this expression and also indicated upregulation in FL. The *SNAKIN 2* antimicrobial peptide precursor was found to be up-regulated in response to *A. rabiei* infection. As IC was the most resistant genotype tested in this study, *SNAKIN 2* peptides might be integral to the resistance mechanisms. PRPs are structural proteins of the primary cell wall involved in strengthening of cell wall to restrict pathogen penetration. Significant up-regulation of PRP by 24 hpi in FL might effectively restrict pathogen penetration which was observed to occur at 24 hpi. The expression profiles of superoxide dismutase copper chaperone (SDCC) precursor and glutathione-S-transferase (GST) evidenced oxidative burst in this pathosystem. SDCC was significantly down-regulated at 6 hpi in FL and LA and at 48 hpi in IC. On the other hand, GST was down-regulated only in IC at 72 hpi. The other proteins whose regulation may be necessary for ascochyta blight resistance include several PR-proteins, proline-rich proteins, disease resistance response protein DRRG 49-C polymorphic antigen membrane protein (PAMP) and Ca-binding protein. Comparisons between resistant and susceptible genotypes revealed potential gene signatures predictive of effective *A. rabiei* resistance (Coram and Pang 2006).

Verticillium dahliae causes the destructive disease of cotton. The ERF transcription factor gene (GbERF2) from sea-island cotton following infection by *V. dahliae* was cloned by suppression subtraction hybridization. This gene encodes a predicted polypeptide of 198 amino acids with a MW of 22.5 kDa. The *GbERF2* protein had a highly conserved ERF domain, whereas the nucleotide and amino acid sequences showed low homology with other ERF plant proteins. Constitutive expression of *GbERF2* in leaves and other tissues was revealed by RNA blots. When the plants were exposed to ET treatment or *V. dahliae* infection, rapid accumulation of GbERF2 transcripts occurred. On the other hand, other stresses due to salt and drought resulted in slight accumulation of the *GbERF2* transcripts. The transgenic tobacco plants expressing *GbERF2* constitutively accumulated higher levels of PR gene transcripts like PR-1b, PR-2 and PR-4, whereas the resistance to fungal pathogen *Alternaria longipes* was enhanced in transgenic tobacco plants. But their resistance to bacterial pathogen *Pseudomonas syringae* pv. *tabaci* did not show any improvement. The results indicated that *GbERF2* may have a significant role in response to ethylene stress and infection by fungal pathogens (Zuo et al. 2007)

Microarray and proteomic analyses are the two approaches that hold promise to provide a better understanding of the entire network of host responses to infection by microbial plant pathogens. The usefulness of microarray technology has already been discussed. Both approaches permit a global analysis of cellular regulation. However, the microarray is restricted to the analysis of gene expression. The possibility of monitoring the accumulation and modification of proteins is considered as an advantage of proteomics over microarray. The level of gene expression does not necessarily correlate with the protein levels in a cell. Further, the genes required for a response are not necessarily the same genes that are differentially regulated as

a result of response (Gygi et al. 1999; Birrel et al. 2002). Hence, it is considered that analysis of protein levels and protein modification profiles may indicate more clearly the final players in a cellular response. Proteomics is capable of providing information on the complete set of expressed proteins and/or post-translational modifications at a given time or in a particular tissue. In plants, protein phosphorylation seems to represent a major control mechanism for protein activity and to be an important post-translational modification in response to microbial pathogens. Many signaling components such as kinases and transcription factors (TFs) occur only in very low copy numbers, making it difficult to detect them. Proteomics has frequently been used to seek out over-or under-expression of proteins that are separated by two dimensional polyacrylamide gel electrophoresis (2DE) which can also be coupled with immunodetection of phosphorylated proteins after Western blotting (Thurston et al. 2005).

The proteins that were differentially produced during *Rice yellow mottle virus* (RYMC) infection in two cultivars IR64 (susceptible) and Azucena (partially resistant) were identified. New uncharacterized hypothetical proteins as well as proteins involved in metabolism, stress and protein synthesis were detected. The protein profiles indicated a common signaling network for biotic and abiotic stress (Ventelon-Debout et al. 2004). Another study to monitor the global changes in protein levels in rice in response to *Magnaporthe grisea* causing blast disease as well as to salicylic acid (SA), jasmonic acid (JA) and H_2O_2 was taken up. Some of the proteins differentially expressed due to pathogen infection were also affected by JA treatment, suggesting the existence of a similar or common signaling pathway. The investigation indicated the usefulness of proteomic approach where the interplay of response pathways such as pathogen and JA pathways may be monitored (Kim et al. 2003b). The steady-state level of proteins, as well as co-and post-translational modifications of proteins can be monitored by using proteomics. A generally conserved signaling pathway have been shown to be involved in the perception of a variety of microbial elicitors (Peck et al. 2001).

To have an insight into plant pathogenesis, it is necessary follow cytosolic and nuclear protein phosphorylation and also those proteins in the plasma membrane (PM). The PM proteins are known to be involved in the perception of elicitors in regulating early responses and are often the targets of pathogen signals. The plasma membrane has been found to be one of the most common sites for plant cells to perceive signals from the invading pathogens. The products of many *R* genes are membrane-associated receptors for the interactions of plant cells with pathogen-derived elicitors (Martin et al. 2003). The identification of signaling processes and phosphoproteins at the PM is of particular interest in plant-microbe interactions. Proteomic research may enable researchers to overcome an important bottle neck in signaling studies, which is the detection of in vivo phosphorylation sites of low abundance membrane proteins that are very difficult to identify by classical methods (Thurston et al. 2005).

Plant genomes respond to specific pathogen signals and trigger a hypersensitive response (HR) following recognition of pathogen presence by R proteins encoded by cluster of *R* genes. Later concomitant with the HR at the site of infection,

development of systemic acquired resistance (SAR) results in restriction of the spread of pathogens and consequently systemic infection of tissues far away from the site of infection. SAR is a general defense mechanism conferring long-lasting resistance against a broad spectrum of pathogens. Several molecular changes due to SAR induction including accumulation of PR-proteins with antimicrobial properties are observed. In *Arabidopsis*, PR gene induction required the signal molecule salicylic acid (SA) and the transcriptional coactivator NPR1 (nonexpressor of *PR* genes 1, also known as NIM1) (Durrant and Dong 2004). In the *npr1* mutant, SA-induced PR gene expression and SAR were entirely abolished, indicating the involvement of NPR1 in SAR development. Transcription of PR genes in *Arabidopsis* was regulated by the coactivator NPR1 and the repressor SNI1. Pathogen infection triggered an increase in somatic DNA recombination which might result in transmission of changes to the offspring of infected plants. In the *sni1 npr1* double mutant, PR gene induction and SAR were restored. This finding suggested that besides NPR1 and SNI1, additional SA-dependent regulatory components controlling PR gene expression may be present. In order to identify other components, a genetic screen for suppressors of *sni1* was carried out. The *ssn1* (suppressor of sni1) mutant was found to have a recessive mutation in the *RAD51D* gene. SNI1 and RAD51D regulated both gene expression and DNA recombination. RAD51D was shown to be required for NPR1-independent PR gene expression. As a result, the *rad51d* mutant showed enhanced susceptibility to disease. In addition to altered PR gene expression, *rad51d* plants were hypersensitive to DNA damaging agents and were impaired in homologous recombination. The dual role of RAD51D and SN1 in PR gene transcription and DNA recombination suggested a mechanistic link between short-term defense response and long-term survival strategy (Durrant et al. 2007).

The roles of different defense signal compounds for resistance (*R*) gene-mediated resistance such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) have been studied in dicot plants. Accumulation of SA and transient emission of ET have been observed during formation of HR lesions (HRLs) (Gaffney et al. 1993; Delaney et al. 1994). However, SA was not considered to be essential for Cf-2-and Cf-9-dependent resistance of tomato to *Cladosporium fulvum* (Brading et al. 2000). No increase in SA levels was discernible in upper leaves of rice plants in which resistance to *Magnaporthe oryzae* causing blast disease, was induced by preinoculation of lower leaves with *Pseudomonas syringae* D20 (Silverman et al. 1995). Rice plants with *Pi-i* R-gene mediated resistance to blast disease also did not exhibit increase in levels of SA, suggesting that SA may not function as a critical defense signal for induced or *R*-gene-mediated resistance. However, SA may play an important role in modulating the redox balance, protecting rice plants from oxidative stress (Yang et al. 2004). In rice-blast pathosystem, neither alterations in the endogenous jasmonic acid (JA) level following infection nor induction of local resistance to blast in rice plants treated with JA could be observed (Schweizer et al. 1997). But application of JA prior to inoculation with *M. grisea* resulted in SAR (Schweizer et al. 1998).

The contribution of ET emission to resistance against rice blast disease was studied using susceptible wild-type Nipponbare and its isogenic resistant line IL7 which

contains *R* gene *Pi-i* to *M. grisea* race 003. Small necrotic lesions due to HR were formed in resistant IL7 leaves at 42 to 72 h postinoculation (hpi), while whitish expanding lesions were seen in susceptible wild type leaves. Enhanced ET emission at 48 hpi accompanied by increased 1-aminocyclopropane-1-carboxylic acid (ACC) levels and highly elevated ACC oxidase (ACO) activity in IL7 leaves was observed. In contrast, an enhanced ACC increase at 96 hpi was seen in wild-type susceptible leaves. Of the six ACC synthase (ACS) and seven ACO genes present in rice genome, OsACS2 was transiently expressed at 48 hpi in IL7 and at 96 hpi in wild-type and OsACO7 was expressed at 48 hpi in IL7. Treatment of rice plants with aminoxyacetic acid (AOA) inhibited ET emission drastically and broke down resistance in IL7 resulting in the formation expanding lesions instead of hypersensitive response lesions (HRLs). The effect of AOA could be reversed by exogenous supply of ACC to IL7 and breakdown of resistance was prevented. Characterization of the rice ACS and ACO gene families showed that enhanced ET emission during formation of HRLs may be supported by specific ACS and ACO genes at the transcriptional level. The results indicate that ET biosynthesis, but not ET itself, is essential for *R*-gene-mediated resistance in rice to blast disease (Iwai et al. 2006).

The phytohormones salicylic acid (SA) and jasmonic acid (JA) have been demonstrated to have a role in regulating signal pathways of plant defense responses to microbial infection and other biotic and abiotic stresses (Creelman and Mullet 1997; Alvarez 2000). JA has been shown to induce pathogenesis-related (PR) proteins and phytoalexins after treatment with elicitors. The responses to necrotrophic fungal pathogens like *Pythium* are regulated by JA pathways (Vijayan et al. 1998). Both positive and negative interactions between SA and JA signaling pathways have been suggested. Expression of SA-dependent (acidic) PR-proteins was inhibited by exogenous application of JA on tobacco leaves. In contrast, the treatment enhanced the expression JA-dependent (basic) PR-proteins (Niki et al. 1998). Enhanced SA-mediated defense responses in JA-insensitive mutants of *A. thaliana* have been reported (Petersen et al. 2000). Defense responses of pepper (chilli) cultivar SCM334 resistant to *Phytophthora capsici* causing *Phytophthora* blight disease were assessed. Expression patterns of HR-related and JA synthesis genes in resistant and susceptible pepper cultivars were analyzed. Catalase (CAT) and peroxidase (POD) are reductases of H_2O_2 that is generated during HR. Expression of the two genes was induced in susceptible cultivar after inoculation and prior to symptom development or penetration of host tissue by the pathogen. On the other hand, expression of these genes was either delayed or insignificant in SCM334 following inoculation with *P. capsici*. The gene *OPR3* participates directly in the octadecanoid pathway for JA (Strassner et al. 2002). *OPR3* mRNA was detected immediately after inoculation of SCM334 leaves, but the levels of JA in mock-treated SCM 334 leaves did not show any increase. Expression of *OPR3* mRNA was accompanied by JA accumulation with a time lag which might be due to processing prior to *OPR3* participation in JA synthesis. The early appearance of JA and later accumulation of SA following inoculation of the resistant cultivar suggested that JA and SA may play different, separate roles in host plant defense response resulting in HR-mediated cell death (Ueeda et al. 2006).

The pathogen-inducible rice *OsAOS2* gene, encoding allene oxide synthase, a key enzyme in the JA biosynthetic pathway was characterized. Exogenous application of JA activated defense expression and local induced resistance in rice seedlings against *M. grisea*. The role of endogenous JA in rice defense response through transgenic manipulation of the JA biosynthesis was examined. The basal level of *OsAOS2* expression was very low in leaves but relatively high in the sheath, culm and flower of rice plants. It is of interest to note that the expression of *OsAOS2* in rice leaves could be induced significantly by infection of *M. grisea.* Accumulation of *OsAOS2* transcripts and higher levels of JA especially after infection of *M. grisea* was observed in transgenic rice leaves carrying the *OsASO2* transgene under the control of a strong, pathogen inducible PBZ1 promoter. In addition, enhanced activation in transgenic lines of *PR*-genes such as PR1a, PR3 and PR5 and higher level of resistance to *M. grisea* infection were also detected. The results suggest a significant role for SA in induction of *PR* genes and subsequent blast resistance in rice plants (Mei et al. 2006).

Protein phosphorylation plays a pivotal role in signaling pathways linking the perception of infection by microbial pathogens and elaboration of defense responses. Protein kinases of different families seem to be involved in defense mechanisms at different cellular levels, including elicitor recognition, as extra cellular or intracellular receptors, signal transduction and introduction of transcriptional activation. In eukaryotes, mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling molecules and they function downstream of sensors/receptors. The signals generated by the sensors/receptors are converted into cellular responses by MAPKs. Several distinct MAP kinase cascades have been shown to be either positively or negatively involved in both *R*-gene-mediated and elicitor-induced disease responses in plants (Suzuki 2002). The MAP kinases are activated by MAP kinase (MAPKK), through dual phosporylation of threonine and tyrosine residues of a TXY motif. Isolation of MAP kinase genes and generation of specific antibodies against their protein products have made it possible to characterize several MAP kinases involved in defense mechanisms. Elicitor-activated ERM kinases from parsely and SA-induced SIP kinase have provided the basic information on the activities of these enzymes (Sesssa and Martin 2000). In parsely cells, an oligopeptide elicitor derived from *Phytophthora sojae* binds to a plasma membrane receptor and activates a wide array of defense responses (Nurnberger et al. 1994). The rapid and transient activation of the MAP kinase, ERMK by the same elicitor was demonsrated later by Ligterink et al. (1997).

The MAPK from tobacco WIPK was activated only in cells carrying the *N* disease resistance gene offering protection to *Tobacco mosaic virus* (Zhang and Klessig 1998a). The SA-induced SIPK activity was induced by various biotic and abiotic elicitors in both host and non-host resistance mechanisms. Rapid and transient activation of SIPK was recorded after nonspecific elicitation in tobacco suspension cells by SA (Zhang and Klessig 1997) and elicitors from *Phytophthora* spp. (Zhang et al. 1998) and leaves by SA and wounding (Zhang and Klessig 1998b). SIPK elicitation in tobacco occurred in a Cf-9-dependent manner after application of the avirulence gene product Avr9 (Romeis et al. 1999). The SIPK activation in

all these treatments was shown to be due to post-translational event(s) and was not associated with an increase in mRNA or protein levels (Romeis et al. 1999).

Pathogen/elicitor-induced activation of SIPK in tobacco or its orthologs in other plant species may be observed within 1 to 5 min representing one of the earliest responses in plants after perception of invading pathogens. Pathogens/elicitors causing HR cell death have been shown to induce long-lasting activation of SIPK, whereas elicitors incapable of causing cell death such as fungal cell wall elicitors are able to activate SIPK only transiently (Zhang et al. 1998). Correlative biochemical analyses and inhibitor studies have revealed that SIPK and WIPK are involved in regulating HR cell death in tobacco disease resistance (Zhang and Klessig 2001).

A plant MAP kinase cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) responds to both fungal and bacterial pathogens. The JA-dependent *PDF1.2* and *TH 12.1* gene activation in *Aradiopsis* was suggested to occur through MEKK1 and At-MPK4 (Asai et al. 2002). Negative regulation of SA-mediated responses by AtMPK4 was reported by Zhang and Klessig (2001). The existence of differential regulation of MAPKs during JA and SA-mediated responses may be detected in different pathosystems. A defense MAP kinase cascade leading to an incompatible plant-microbe interaction also may be differentially regulated during the early phase of a compatible interaction. In a later investigation, the involvement of a MAPK pathway in *Mycosphaerella pinodes* elicitor-induced defense response in pea was investigated. An MAPKK inhibitor (suppressor) was found to interrupt the elicitor-induced MAPK cascade(s) in pea (Uppalapatti et al. 2004).

The tobacco MAPK, Ntf4 shares 93.6% and 72.3% identity with two other tobacco MAPKs SIPK and WIPK respectively. Ntf4 has been reported to be developmentally regulated and function in pollen germination and expressed in developing embryos and seeds. SIPK has a specific role in regulating the ET production in plants under stress (Kim et al. 2003a; Liu and Zhang 2004). Universal expression of SIPK in leaf, stem, root, pollen, anther, pistil, ovary and flower petal was observed in a later investigation. Further, Ntf4 was also found to be expressed in tobacco leaves and suspension cultured cells. Cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea* induced a long-lasting activation of Ntf4 which correlated with a rapid HR-like cell death. Ntf4 alone may be sufficient to cause HR-like cell death, as shown by conditional gain-of-function analysis. Transgenic *Ntf4* plants with elevated levels of Ntf4 protein exhibited accelerated HR cell death when treated with cryptogein. The results demonstrated that Ntf4 is multifunctional. In addition to its role in pollen germination, Ntf4 can also regulate pathogen-induced HR cell death in tobacco (Ren et al. 2006).

The WRKY proteins comprise a family of plant-specific zinc-finger-type factors implicated in the regulation of genes associated with pathogen defense. In vitro, these proteins bind specifically to functionally defined TGAC-containing W-box promoter elements of pathogenesis-related class 10 (PR-10) genes. The cDNA encoded a putative polypeptide of 172 amino acids, containing a single WRKY domain. *St-WRKY1* was strongly induced by *Erwinia carotovora* subsp. *atroseptica* (*Eca*) culture filtrate, indicating this gene to be an elicitor-induced gene. But SA, MeJ, ET or wounding did not induce this gene. However, the treatment with culture

filtrates from recombinant *Escherichia coli* containing plasmids expressing *Eca* pectate lyase genes, *pelB* and *pelD,* resulted in the upregulation of *St-WRKY1*. The results suggest that either proteins encoded by these genes or oligogalacturonides generated by their activity may elicit a potato defense pathway associated with *St-WRKY1* (Dellagi et al. 2000). WRKY70, a unique member of the WRKY transcription factor family has been shown to be an important regulation component in plant defense (Li et al. 2004). WRKY70 is an activator of SA-dependent defense genes and seems to repress JA-regulated genes suggesting that WRKY70 might regulate the balance between JA- and SA-mediated defense and fine-tune the plant resistance response to effectively combat specific pathogens (Li et al. 2004).

A large number of WRKY genes (109) has been identified in the rice genome. However, the functions of most of these genes are still unknown. The *OsWRKY13* gene appears to have an important role in the resistance of rice to two important diseases – blast and bacterial blight diseases. Overexpression of *OsWRKY13* enhanced the levels of resistance at both seedling and adult stages to both diseases and it did not have any effect on plant fertility. Activation of SA synthesis-related genes and SA-responsive genes was induced by overexpression of *OsWRKY13*. In contrast, suppression of jasmonic acid (JA) synthesis-related genes and JA-responsive genes was observed due to overexpression of this gene. OsWRKY protein bound to the promoters of its own and at least three other genes in SA- and JA-dependent signaling pathways. Pathogen infection influenced the binding activity of this protein. The results suggest that OsWRKY mediates rice resistance, as an activator of SA-dependent and suppressor of JA-dependent pathways, by directly or indirectly regulating expression of a subset of genes acting both upstream and downstream of SA and JA (Qiu et al. 2007).

The role of WRKY70 in modulating the balance between plant defense pathways was determined by using two fungal pathogens *Alternaria brassicola* and *Erysiphe cichoracearum* with distinctly different pathogenicity strategies. Crosstalk between signal transduction pathways is an important characteristic of tightly regulated plant defense signaling network. Recognition of the type of pathogen or pathogen-derived elicitor determines the potential synergism or antagonism between defense pathways. Gain or loss of function of WRKY70 caused opposite effects on JA-mediated resistance to *E. cichoracearum.* Up-regulation of WRKY70 resulted in enhanced resistance to *E. cichoracearum*. However, it compromised the plant resistance to *A. brassicola.* Conversely, down-regulation or insertional inactivation of WRKY70 impaired plant resistance to *E. cichoracearum.* The JA-signaling was found to be essential for *Arabidopsis* resistance to *A. brassicola*. This resistance, however, was drastically compromised by over-expression of WRKY70, but enhanced in the *wrky70* mutant background, indicating that WRKY70 could suppress JA-mediated defenses. The results showed that WRKY70 down-regulated both JA- and *Alternaria*- induced genes such as the JA responsive molecular markers *PDF 1.2* and *AtCLH1*. The microarray analysis confirmed the WRKY70-mediated suppression of JA dependent defense pathways (Li et al. 2006).

The physical and functional interactions between structurally related and pathogen-induced WRKY18, WRKY40 and WRKY60 transcription factors in *A thaliana*

were studied. Single WRKY mutants exhibited no or small alterations in response to *Pseudomonas syringae* and *Botrytis cinerea*. However, *wrky18, wrky40* and *wrky18 wrky60* double mutants and *wrky18, wrky40 wrky60* triple mutant showed higher level of resistance to *P. syringae.* But they were more susceptible to *B. cinerea* compared to wild-type plants. The three WRKY proteins seem to have partially redundant roles in plant responses to the two distinct types of pathogens with WRKY 18 playing a more important role than the other two proteins. The contrasting responses of these WRKY mutants to the two pathogens correlated with opposite effects on pathogen-induced expression of SA-regulated *PATHOGENESIS–RELATED 1* and jasmonic acid-regulated *PDF 1.2*. The constitutive expression of *WRKY18* increased resistance to *P. syringae*. But its co-expression with *WRKY40* or *WRKY60* enhanced susceptibility to both pathogens. These three WRKY proteins interact both physically and functionally in a complex pattern of overlapping, antagonistic and distinct roles in plant responses to different types of microbial pathogens (Xu et al. 2006).

The pathogenesis-related (PR) proteins are the proteins coded for by the host plant, but induced only in pathological or related situations (Antoniw et al. 1980). PR-proteins have a role in the resistance response of host plants, particularly in the development of acquired resistance following the production of necrotic lesions. Induction of PR-proteins is associated with the development of systemic acquired resistance (SAR) in tissues/organs far away from the site of inoculation (Ward et al. 1991). A large group of PR-proteins has been shown to be rapidly and massively induced both locally around infection sites and systemically as well (Kombrink and Somssich 1997). The PR-genes and proteins are widely used as marker genes/proteins to study the defense mechanisms of plants. Currently PR-proteins have been classified in the 14 families and at the 6th International Workshop on PR-proteins, three more new families have been proposed (Van Loon and Van Strien 1999; Jwa et al. 2006).

The major acid PR-proteins (PR-1 to PR-5) have been found to be localized in the intercellular space of leaf tissues where contact between PR-proteins and pathogens may occur. The biochemical function of PR-1, the most abundant PR protein is not clear. The suggested functions include prevention of virus diffusion, restriction of fungal invasion and protection against environmental stress. Several basic PR proteins are known to have antifungal activity (Nidermann et al. 1995). Two basic PR-1 proteins from barley leaves infected by *Blumeria graminis* f.sp. *hordei* (*Bgh*) were isolated (Bryngelsson et al. 1994). The subcellular localization of a basic PR-1 barley was investigated by applying immunocytochemical methods. PR-1 protein accumulated on the outer electron dense layer of the cell wall of primary hyphae of *Bipolaris sorokiniana*. In addition, presence of PR-1 in the cytoplasm of primary hyphae, junctions between host cells and in host cell wall appositions was also detected. But no PR-1 labeling could be observed on the secondary hyphae (Santém et al. 2005).

Localization of chitinase (CA Chi2) in the compatible interactions of pepper stems with *Phytophthora capsici* was visualized by employing immunogold labeling technique. In the incompatible interaction, the quantity of gold particles in the fungal cell surface was limited, but even distribution of gold particles over the entire

fungal wall which was in intimate contact with host cell wall, could be observed. In contrast, most of the gold particles were seen over the cell wall area of the pathogen interacting with resistant tissues. Degradation of fungal cell wall was evident at the hyphal tips which had dense depositions of gold particles indicating that the activity of the host chitinase on the pathogen is limited to the cell wall, since the cytoplasm of fungal cell was nearly free of gold labels (Lee et al. 2000). The defense gene response of grapevine inoculated with *Botrytis cinerea* were assessed by immunodetection methods. Newly synthesized acid proteins (4) that cross reacted with a tobacco PR-2a (β-1,3-glucanase) antiserum were detected. Strong similarities between one of these isoforms with β-1,3-glucanases from other plant species were demonstrated (Renault et al. 2000).

PR-proteins belonging to several families have been demonstrated to possess antimicrobial properties, thus may have possible role in plant defense against pathogens. PR-1 and PR-5 [thaumatin-like proteins (TLPs) and osmotins] which are considered to create transmembrane pores and hence were designated permatins; PR-2 (β-1,3-glucanases) and PR-3,4,8 and 11 (chitinases) attack β-1,3-glucans and chitin respectively constituing components of the cell walls in most higher fungi. Transgenic plants overexpressing some PR-genes exhibited enhanced levels of resistance to fungal pathogens (Grison et al. 1996; Punja and Raharjo 1996; Yamamoto et al. 2000). Chitinase and glucanase proteins purified from the leaves of resistant grapevine cultivar inhibited the growth of *Uncinula necator* in vitro (Giannakis et al. 1998). Likewise, the mycelial growth of *B. cinerea* was inhibited by grapevine chitinase and osmotin (Salzaman et al. 1998). In a later study, osmotin and thaumatin-like protein were found to possess strong antifungal activity and display a synergistic effect when these PR-proteins were present together. The growth of *Phomopsis viticola* and *B. cinerea* was arrested in addition to inhibition of spore germination and germtube elongation of the grape pathogens. Induction of chitinase and β-1,3-glucanase in grapevine leaves inoculated with *B. cinerea* was demonstrated. Enhanced level of transcription of a chitinase (PR-3), a glucanase (PR-2) and TLP (PR-5) gene was detected in grapevine leaves and preveraison berries infected by *U. necator* (Renault et al. 1996; Jacobs et al. 1999; Monteiro et al. 2003).

The role of intercellular washing fluid (IWF) from leaves of apple (*Malus domestica* cv. Elstar) infected with a cloned isolate of *Venturia inaequalis* causing scab disease was investigated. Variations in the composition of proteins in the IWF were assessed using SDS-PAGE and two dimensional electrophoresis techniques during and after infection by this fungal pathogen. β-1,3-glucanase, chitinase, thaumatin-like protein(TLP) and a cysteine-like protease were detected in *M. domestica* leaves infected by *V. ianequalis*. Immuno-blotting with specific antibodies detected some of the PR-proteins. The protein profiles of IWF from apple lines showing resistance to scab, powdery mildew and fire blight diseases showed some similarity, indicating the constitutive production at least of some of the PR proteins in resistant cultivar Elstar (Gau et al. 2004).

Plants possess downstream defense genes, such as enzymes involved in the generation of phytoalexins, the enzymes of oxidative stress protection, tissue repair and

lignification and small peptides with antimicrobial activity, like thionins, defensins and lipid transfer proteins, in addition to *R* genes and genes encoding transduction proteins. Furthermore, the genes encoding a large group of PR-proteins are also induced. The expression of PR-proteins encoding genes and genes involved in the phenylpropanoid metabolism was determined in grape leaves of susceptible cv. Riesling (*Vitis vinifera*) and resistant cv. Gloire de Montpellier (*V. riparia*) in response to inoculation with host-pathogen *Plasmopara viticola* and nonhost pathogen *Pseudoperonospora cubensis* (infecting cucumber). The chitinase genes *PR-3* and *PR-4* were constitutively expressed in Gloire, possibly involved in preformed resistance mechanism. On the other hand, the β-1,3-glucanase gene (*PR-2*) was exclusively expressed after challenge with the pathogen. In Gloire, expression of PR-2 occurred immediately after inoculation with non-pathogen as compared to the plants inoculated with *P. viticola.* An earlier activation of the resistance response when inoculated with non-pathogen may be due to a better and more rapid recognition of *P. cubensis.* Since chitin and glucan are present in the cell walls of these two pathogens (Peronosporaecae), the constitutive or induced expression of chitinase and glucanase encoding genes may inhibit either directly degrading pathogen cell walls or produce fragments of cell wall, which can provoke as elicitors, the activation of downstream processes. Expression of defense-related genes in Riesling was influenced primarily after inoculation with *P. cubensis* such as *PR-2, PR-3, PR-4,* a *PGIP* gene and also genes encoding enzymes involved in anthocyanin biosynthesis, *DFR, F3H* and *LDOX* was altered. *PR-5* was shown to be constitutively expressed in Gloire and Riesling as revealed by RT-PCR assessment. *PR10* transcript accumulation was also seen after pathogen challenge. In Riesling, *PR-10* was clearly induced at 2 hai by both pathogens (Kortekamp 2006).

Accumulation of phytoalexins is the most commonly elicited defense response in grapevine plants. Very high concentrations of stilbenic phytoalexins were observed at the site of infection in resistant cultivars artificially inoculated with *P. viticola.* The motility of the zoospores was drastically reduced leading to restricted disease development (Pezet et al. 2004). Synthesis of stilbenes occurred if *PAL* and other genes were induced (Jeandet et al. 2002). However, PAL appeared to be constitutively expressed in the greenhouse plants of both *Vitis* spp., but its expression was totally repressed in the affected tissue following inoculation with *P. cubensis.* The expression of the genes *DFR*, *F3H, LDOX* and *FS* required for synthesis of proanthocyanidins, the precursors of tannins, was enhanced in Riesling after inoculation with *P. cubensis.* In Gloire, LDOX and FS were not expressed after inoculation with nonpathogens. Rapid activation of gene expression and production of defense-responsible compounds results in enhanced or induced resistance (Kortekamp 2006).

Chitin is one of the major components of fungal cell walls. Plants produce chitinases following infection by pathogens. Chitooligosaccharides may be generated from the cell walls of pathogenic fungi by the action of endochitinases and they are able to elicit strong defense responses in some plant species (Shibuya and Minami 2001). In chitooligomer-treated *A. thaliana* seedlings, the transcript levels for 71 ESTs, representing 61 genes registered dramatic increases (>3 folds)

and chitin induced defense responses proceeded through an as yet uncharacterized signaling pathway (Ramonell et al. 2002; Wan et al. 2004). High-affinity binding sites for chitooligosaccharides were detected in suspension cultured rice and tomato cells. A 75-kDa chitooligosaccharide-binding protein was identified in rice plasma membranes by affinity labeling and cross-linking (Knogge and Scheel 2006). The response of *A. thaliana* seedlings to chito-octamars and hydrolysed chitin was studied by using the ATH1 Affymetrix microarrays consisting of about 23,000 genes. Microarray expression profiles of several genes were determined, via northern analysis and quantitative RT-PCR. The T-DNA insertion mutants for nine chitooligomer responsive genes were more susceptible to powdery mildew pathogen *Erysiphe cichoracearum* than wild-type. The three mutants had mutation in genes for two disease resistance-like proteins and putative E3 ligase. The loss-of-function mutants that were highly susceptibile were considered to provide direct evidence that the chito-octamer is an oligosaccharide elicitor of plant defenses (Ramonell et al. 2005).

Chitinases may participate in a broader stress response of the plants. The presence of a Class III chitinase was examined by immunological techniques in the root and stem base of a susceptible (Galia) and resistant (Bredor) cultivars of melon during infection by *Fusarium oxysporum* f.sp. *melonis (FOM).* Using the specific antibody, the distribution of a class III chitinase was monitored by both Western blotting analysis and by immunolocalization. The constitutive expression of chitinase III in stem base tissues of Galia was determined by Western blotting. No chitinase band was detected in resistant cultivar. But in Bredor plants that were wounded or infected by *FOM* three bands at 13, 25 and 30 kDa were detected. On the other hand, following infection, in Galia plants, the bands at 30 and 35 kDa became faint. Chitinase deposition was more intense in Bredor plants in which pathogen hyphae were not observed. But in susceptible plants the presence of hyphae in the xylem vessels could be noted and very faint chitinase expression was evident. It appears that for melon resistance to *FOM*, not only the total chitinase III present, but rather the contribution of some of its isoforms is important as shown by Western blotting. The results strongly suggested a relationship between chitinase III and melon resistance to *FOM* (Baldé et al. 2006).

In order to understand the molecular machinery involved in the perception and transduction of chitin oligosaccharide elicitor, a high-affinity binding protein for this elicitor was isolated from the plasma membrane of suspension-cultured rice cells. This protein designated CEBip was characterized and the corresponding gene was cloned. CEBip was shown to be a glycoprotein consisting of 328 amino acid residues and glycan chains. The *CEBip* gene was knocked down by RNA interference which resulted in the suppression of elicitor induced oxidative burst as well as the gene responses. This finding indicated that CEBip has a pivotal role in the perception and transduction of chitin oligosaccharide elicitor in the rice cells. The presence of two LysM motifs in the extracellular portion of CEBip was indicated by the structural analysis of CEBip. The putative Nod-factor receptor kinases involved in the *Rhizobacterium*-plant symbiotic relationship, was also reported to have the LysM motif. The results indicated the involvement of partially homologous plasma

membrane proteins both in defense and symbiotic signaling in plant cells (Kaku et al. 2006).

The PR Class I (PR-1), a dominant group is frequently used as a marker for systemic acquired resistance (SAR). In rice, induction of a basic PR1 (OsPR1b) protein in leaf infected by the blast disease pathogen *Magnaporthe grisea,* was revealed by immunoblotting using an antibody specific to the major basic PR-1 of tomato (Schweizer et al. 1997). The antibody raised against the 17-kDa OsPR-1a protein cross-reacted also with two additional proteins (ca. 19 and 22-kDa) present in and around blast lesion (Rakwal et al. 2001). The two *OsPR1* genes were differentially regulated by diverse signals such as pathogen infection, wounding and chemicals (Agrawal et al. 2000, 2001). The family of PR2 proteins, β-1,3-glucanases are present abundantly and they are highly regulated enzymes widely distributed in seed-plant species. They exist as multiple structure isoforms that differ in size, isoelectric points, primary structure, cellular localization and pattern of regulation. Remarkable synergistic effect resulting in significant reduction of susceptibility of plants to infection by fungal pathogens has been demonstrated in plants expressing β-1,3-glucanases transgenes alone or in combination with chitinase transgenes (Zhu et al. 1994; Lusso and Kuć 1996). Over expression of *OsGns1* under the control of the CaMV 35S promoter in rice imparted resistance against *M. grisea*, accompanied by HR and enhanced PR protein expression (Nishizawa et al. 2003).

The rice *OsPR3* chitinase cDNA (RC7) was isolated from rice plants inoculated with *Rhizoctonia solani* and this gene was transgressed into *indica* rice cultivars IR72, IR64, IR68899B, MH63 and Chinsurah Boro II. These cultivars are useful for the control of rice sheath blight disease (Datta et al. 2001). The accumulation of a novel *OsPR-4* a single copy gene in compatible and incompatible host-pathogen interactions was observed (Agrawal et al. 2003). Over expression of *OsPR5* gene was reported to increase resistance to sheath blight disease (Datta et al. 1999). Induction of *OsPR5* could be recorded in rice leaves and leaf sheaths treated with JA and copper and also in leaf sheaths and roots of 2-week-old rice seedlings and in mature plants infected by *M. grisea,* by employing a specific anti-OsPR5 polyclonal antibody (Rakwal et al. 2001). The proteinase inhibitors (PIs) belonging to the PR6 family are widely distributed in the plant kingdom. Two novel PI genes, *OsBBPI* and *OsPIN* in rice leaves were identified. The gene OsPIN in rice leaves were identified. The gene *OsPIN* was induced in abundance in a compatible, but not in incompatible interaction. Hence it is considered that *OsPIN* may be used as a marker for studying the signaling pathway triggered in rice-*M. grisea* pathosystem (Agrawal et al. 2002). Induction of PR10 proteins, an ubiquitous class of intracellular (other PR proteins being intercellular) defense-related proteins were first described in cultured parsley cells following treatment with elicitor (Somssich et al. 1988). In rice infected by *M. grisea*, the *RPR10a* and *RPR10b* genes were up-regulated, *RPR10a* being expressed most strongly in a localized fashion (McGee et al. 2001).

Resistance to *Aspergillus flavus* infecting maize kernels was considered to be associated with kernel proteins. A 14-kDa trypsin inhibitor (TI) protein was constitutively expressed in mature kernels of resistant maize genotypes at higher levels compared to susceptible lines which showed low or no expression of TI protein

(Chen et al. 1998). TI protein had strong inhibitory activity against *A. flavus* in vitro (Chen et al. 1999). In a later study, a constitutive kernel endosperm protein was identified by comparison of proteomics of three resistant and three susceptible maize genotypes. Expression of this protein, identified as PR-10 protein, was five fold higher in resistant lines than in susceptible lines. The corresponding cDNA (*pr-10*) was cloned and sequenced. This *pr-10* encoded a protein of 160 amino acids with a predicted molecular mass of 16.9 kDa. The expression of *pr-10* during kernel development increased five fold between 7 and 22 days after pollination. Further, the expression of *pr-10* was induced by infection with *A. flavus* only in resistant genotypes but not in the susceptible ones. The maize Zm PR-10 expressed in *Escherichia coli* had RNase and antifungal activities. Leaf extracts of transgenic tobacco plants expressing maize *pr-10* also possessed RNase activity in addition to inhibitory action on the growth of *A. flavus*. The results suggested that ZmPR-10 might have a role in kernel resistance to *A. flavus* (Chen et al. 2006b).

The mechanisms of differential regulation of the activation of potato defense genes in response to infection by two genotypes, US-1 and US-8 of *Phytophthora infestans* were studied. PAL (phenylalanine ammonia-lyase) and HMGR (3-hydroxy, 3-methylglutaryl COA reductase) are the key enzymes involved in the phenylpropanoid and terpenoid pathways respectively. They are considered to have a role in disease resistance mechanism in many plants (Kervinen et al. 1998; Kim et al. 2004a). Differential expression of *Pal1* and *hmgr2* was investigated by applying northern blot analysis in two potato cvs. Russet Burbank (RB) and Kennebec (KB) susceptible and moderately tolerant respectively following inoculation with US-1 and US-8 isolates of *P. infestans.* The accumulation of *pal1* transcripts was found to be less in response to US-8 as compared to US-1 and occurred earlier in KB than in RB. This was attributed to stronger defense gene suppression by US-8 isolate. No apparent strong accumulation of *hmgr2* transcripts could be noted in RB as compared to KB inoculated with either US-1 or US-8 isolates. As the expression of these two genes was stronger in proximal and distal leaflets when compared to the local site of inoculation, it was suggested that molecular signals may be translocated from the site of inoculation to other healthy parts of the potato plants (Wang et al. 2004c).

Phytoalexins are antimicrobial compounds synthesized de novo by plant tissues as a defense response following infection by plant pathogens. As resistance in carnation to *Fusarium* has been shown to be polygenic (Baayen et al. 1991), additional resistance factors other than anthranilic acid-derviative phytoalexins (AADP) may also be involved. Furthermore, the presence of carnation AADP has not been detected in the roots of resistant genotypes, the natural sites of infection by soilborne *F. oxysporum* f.sp. *dianthi (Fod).* Protective molecules constitutively present in healthy plant tissues are designated 'phytoanticipins'. A constitutive fungitoxic flavonol triglycoside was isolated from the roots and stem of the most resistant carnation cv. 'Novada' which was found to be an active producer of AADP (Curir et al. 2001). In a further study, the biosynthesis of this flavonol phytoanticipin, kaempferide 3-0-[2^{G}-β-D-glucopyranosyl]-β-rutinoside, was stimulated by inoculation with *Fod* like the phytoalexin. The results suggest that the levels of some

preformed antifungal falvonoids may be significantly enhanced following infection and these fungitoxic molecules may actively cooperate with compounds that are synthesized postinfectionally, resulting in higher degree of resistance to the invading fungal pathogen (Curir et al. 2005).

Tomato plants contain α-tomatine (phytoanticipin) a steroid glycoalkaloid, at concentrations that may inhibit fungal infection in leaves, stems, roots, flowers and green fruit (Arneson and Durbin 1968). The fungal pathogens producing tomatinase that detoxifies α-tomatine are able to colonize the tomato tissues (Ruiz-Rubio et al. 2001). *Fusarium oxyxporum* f.sp. *lycopersici* (*FOL*) was able to degrade α-tomatine to less toxic metabolite because of the extracellular enzyme tomatinase. The tomatinase gene (*FoToml*) was also found in certain strains of *F. oxysporum* nonpathogenic to tomato plants. Genes encoding Chi9 and GluB, both of which are intra-cellular and basic PR-proteins, were induced in tomato plants inoculated with non-pathogenic strains. Prior inoculation of tomato plants with non-pathogenic strains which strongly induced *Chi3* mRNA in tomato plants, suppressed the development of the vascular wilt caused by FOL (Ito et al. 2005).

3.2 Bacterial Diseases

3.2.1 Genetic Basis of Resistance

As in fungal diseases, molecular techniques have been applied for characterization of genes involved in conferring resistance to plant bacterial diseases. Monogenic and quantitative resistance have been studied using molecular markers. Many major genes conferring resistance to bacterial blight disease of rice caused by *Xanthomonas oryzae* pv. *oryzae (Xoo*) have been identified and named in a series from *Xa1* to *Xa28*. The resistance genes (>10) have been mapped on various chromosomes, especially 4 and 11 (Kinoshita 1995; Lee et al. 2003; Yang et al. 2003). The wild rice *Oryza longistaminata* is highly resistant to *Xoo.* The *Xa21* locus conferring resistance to all known races of *Xoo* in India and Philippines was transgressed from *O. longistaminata* to rice cv. IR24. The *Xa21* gene coding for a receptor kinase-like protein was inherited as a single gene in transgenic plants (Song et al. 1995; Wang et al. 1995). These plants exhibited resistance to 29 of 32 isolates of *Xoo* from India and other South East Asian countries. The presence of a single member of multigenic family *Xa21* appeared to be sufficient to confer resistance to various isolates of *Xoo* as deduced from the identical spectrum of resistance shown by the transgenic lines and the wild-type (Wang et al. 1996; Ronald 1997). A dominant gene *Xa25(t)* for bacterial blight resistance was identified in Minghui 63, a restorer line used for hybrid rice production in China. This gene conferred resistance in Minghui 63 to *Xoo* strain PXO 339 at both seedling and adult stages. In addition, *Xa25(t)* also partly contributed to the resistance of Minghui 63 to another Chinese Xoo strain J2691 at seedling stage only. *Xa25(t)* was mapped to the centromeric

region of chromosome 12. The genomic location of this gene was found to be similar to blast resistance genes *Pi-ta* and *Pi-ta2* identified earlier (Chen and Chen 2002).

The development of *Xylella fastidiosa* (*Xf*), causing grape Pierce's disease (PD), in resistant and susceptible genotypes representing a diverse selection of *Vitis* spp. was determined by measuring bacterial movement and accumulation. The grapevine entries were artificially inoculated under greenhouse conditions. A double-antibody sandwich enzyme-linked immunoassay (DAS-ELISA) was optimized for quantification of *Xf* populations. At 12 weeks postinoculation, the populations of *Xf* in stem internode, stem node, petiole and leaf blade samples from the different genotypes were estimated by applying the optimized DAS-ELISA procedure. This procedure was critical in measuring quantitative differences in *Xf* population across different plant parts in the resistant genotypes. Differences in *Xf* populations between stems and leaves of resistant grape genotypes suggested that a localized mechanism might operate within xylem and not fully functional or possibly absent in the xylem of petioles and leaf blades. The quantitative ELISA estimates of *Xf* in greenhouse-grown grapevines may be an effective index of the level of PD resistance under field conditions (Krivanek and Walker 2005).

The pepper accession, PM687 exhibited stable resistance to the bacterial wilt disease caused by *Ralstonia solanacearum* (*Rs*). In addition, this accession was also reported to be resistant to *Phytophthora capsici*, *Leveillula taurica* and *Potato virus Y* (PVY). To determine the linkage relationships, genetic analysis of resistance to pepper bacterial wilt disease was performed in the double haploid progeny from across between PM687 and susceptible cv. Yolo Wonder. Two to five genes with additive effects were considered to control resistance to *Rs,* indicating an oligogenic control. Susceptibility to *Tobacco mosaic virus* (TMV) and to nematode *Meloidogyne* spp. were significantly linked with resistance to bacterial wilt. In contrast, resistance to *P. capsici* or *L. taurica* was not linked with susceptibility to virus and nematode. As the genetics of resistance to bacterial wilt in pepper and tomato is similar, comparative mapping of resistance quantitative trait loci may provide useful leads for the better understanding of quantitative trait loci (QTLs) (Lafortune et al. 2005).

Studies to determine the genetic basis for resistance/tolerance of apple genotypes to fire blight disease caused by *Erwinia amylovora* (*Ea*) were taken up. *Malus* spp. including *M. robusta, M. sublobata, M. astrosanguinea, M. prunifolia* and *M. fustta* were reported to be potential sources of resistance to *Ea* (Aldwinckle and Beer 1979). In addition, *Malus* x *domestica* cultivars such as Nova Easygro and Florina were also found to show significant level of resistance to fire blight disease. Quantitative trait loci (QTLs) for apple scab resistance (*Venturia inaequalis*) have earlier been identified (Durel et al. 2003). Hence, QTL mapping approach was applied to study fire blight resistance in apple also. The F_1 progenies of Fiesta x Discovery were inoculated with the Swiss strain *Ea*610 under controlled conditions to identify QTL for fire blight resistance. QTL was identified on the linkage group 7 of Fiesta (F7). F7 QTL was identified with two different strains and hence it was considered to be stable. The results suggest that DNA markers for this QTL

may have genuine potential to be translated into an easy-to-use tool for rapid selection of genotypes with higher level of resistance to fire blight disease (Khan et al. 2006).

By utilizing marker-assisted selection (MAS) procedure, some of the mapped resistance genes have been transferred. High density genetic linkage maps are available including simple sequence repeats (SSR) markers. Three rice bacterial blight resistance genes *Xa1, Xa21* and *Xa26* have been cloned by map-based cloning strategy (Song et al. 1995; Yoshimura et al. 1998; Sun et al. 2004). The gene *Xa2* located on the long arm of chromosome 4 confers resistance to T7147 of *Xoo.* New SSR markers (120) were developed to determine the locus of *Xa2*. The nearest SSR markers to *Xa2* were identified as HZR 950-5 and HZR 970-4. The 190-kb region containing these markers showed the presence of a homologous sequence of LRR-kinase. The results indicated the possibility of transferring or pyramiding *Xa2* by MAS in rice breeding programs and for cloning *Xa2* by map-based cloning in combination with a long-range PCR strategy (He et al. 2006b).

Bacterial spot disease of tomato a complex disease due to four species of *Xanthomonas* and bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) account for appreciable crop loss. The resistance gene *Pto* conferred the HR to race O strain of *Pst.* The locus *Rx3* was found to control up to 41% of the variation for resistance to bacterial spot race T1 in the field trials and it was associated with HR following infiltration. Both *Pto* and *Rx3* were shown to be linked in repulsion phase on chromosome 5. The elite breeding tomato lines Ohio 981205 carrying *Pto* and Ohio 9834 carrying *Rx3* were crossed. Marker-assisted selection (MAS) procedure was employed to the F2 progeny and to $F_{2:3}$ families in order to select for coupling-phase resistance. Based on HR in greenhouse screens using *Pto* race O and bacterial spot race T1 isolates, resistance was confirmed in all families selected. Resistance to bacterial spot race T1 was confirmed in 33 families which were also resistant to bacterial speck underfield conditions indicating the usefulness of MAS procedure (Yang and Francis 2005).

Based on a conventional inoculation method, the levels of resistance of 277 cultivars and 70 wild *Dianthus* accessions to the bacterial wilt disease caused by *Burkholderia caryophylli* were assessed. A highly resistant wild species *D. capitatus* subsp. *andrzejowskianus* was identified and a new bacterial wilt resistant line was developed by interspecific hybridization. Since the conventional breeding method required long time and intensive labor, the bulked segregant analysis (BSA) was used to identify RAPD markers linked to the gene controlling bacterial wilt resistance. A total of 505 primers were screened to obtain RAPD markers useful for selecting resistant carnation lines. By applying BSA, eight RAPD markers linked to a major resistance gene were identified. The marker WG44-1050 showed the greatest effect on disease resistance. This RAPD marker was converted to a sequence-tagged site (STS) marker suitable for MAS. WG44-1050 was specifically amplified by five combination of primers. One specific pair of STS primers developed in this investigation could detect a single, clear DNA fragment tightly linked to a major resistance gene easily and reliably (Fig. 3.4). STS marker had a higher degree of reproducibility compared to RAPD marker (Onozaki et al. 2004) [Appendix].

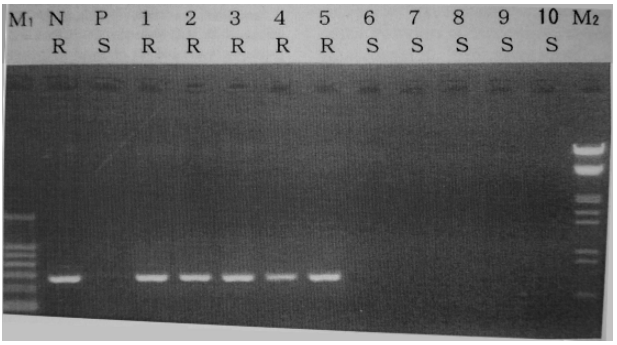

Fig. 3.4 Detection using the specific STS marker generated using a set of primers 44-F80 and 44-R758 for PCR amplification
The marker is present in the resistant parent and in segregants of carnation; they exhibit resistance to bacterial wilt caused by *Burkholderia caryophylli*. (Courtesy of Onozaki et al. 2004; Kluwer Academic Publishers, Netherlands)

3.2.2 Molecular Basis of Resistance to Bacterial Diseases

The microbial plant pathogens, have to successfully breach the protection offered by exterior surfaces of plants and preformed antimicrobial compounds followed by cell walls forming an effective second barrier to invaders that are able to gain entry into interior spaces. Furthermore, the pathogens have to overcome the formidable plant immunity response. Plant immunity comprises of two components operating on different time scales. The basal defense system operates early in the interaction between the host and pathogen, whereas the resistance *R* gene-mediated defense operates on the time scale of hours. The pathogen-associated molecular patterns (PAMPs) that mediate early basal response, include lipopolysaccharide, peptidoglycan bacterial flagellin and yeast mannans (Nurnberger et al. 2004; Pemberton et al. 2005). The innate immune response may be induced also by the bacterial elongation factor EFTu (Kunze et al. 2004). The receptors located in the plasma membrane recognize PAMPs, activating a phosphorylation cascade upon binding, leading to the induction of early basal resistance (Gómez-Gómez and Boller 2002). This basal resistance may be responsible for preventing colonization of nonpathogenic bacteria.

The PAMP-triggered immunity appears to be sufficient to restrict infection before the pathogen becomes established, as demonstrated by work on PAMP flagellin. The connection between inhibition of pathogen growth and the recognition of the PAMP flagellin by the receptor FLS2 was established by Zipfel et al. (2004). Flagellin is the major protein of flagella and also a well characterized PAMP which is recognized by the Leu-rich repeat receptor kinase FLS2 in *Arabidopsis*. FLS2 is located in the plasma membrane and it is considered to be involved in the early bacterial-plant interaction by recognizing and binding flagellin (Dangl and McDowell 2006). Treatment of plants with flg22, a peptide representing the elicitor-active epitope of flagellin induced the expression of several defense-related genes and triggered

resistance to pathogenic bacteria *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC 3000) in wild-type plants, but not in plants carrying mutations in the flagellin receptor gene *FLS2*. This induced resistance appeared to be independent of SA, JA and ET signaling. Wild-type and *fls2* mutants exhibited enhanced resistance when treated with crude bacterial extracts, even devoid of elicitor-active flagellin indicating the existence of functional perception systems of PAMPs other than flagellin. The results indicated that flagellin perception could restrict bacterial invasion, possibly at an early step thus contributing to the plant disease resistance (Zipfel et al. 2004).

The flg22 peptide strongly induced the transcription of *NHO1* in *Arabidopsis*, limiting the *in planta* growth of nonhost *P. syringae* pv. *tabaci* (*Ptab*) strain to which *Arabidopsis* is a nonhost. *NHO1* was induced in a flagellin dependent manner. *Ptab* strain lacking the flagellin gene *flic* induced disease symptoms and multiplied in *Arabidopsis* plants, demonstrating that flagellin signaling contributed to non-host resistance. In contrast to prolonged induction of *NHO1* by *Ptab* strain, *Pst* DC 3000 only transiently induced NHO1 transcription, also in a flagellin-dependent manner. Later *Pst* DC 3000 readily suppressed the *NHO1* induction, whereas the mutant strains defective in TTSS were diminished in their ability to suppress *NHO1*. At least nine effectors HopS1, HopAl1, HOPAF1, HopT1-1, HopT1-2, HopAA1-1, HopF2, HopC1 and AvrPto are capable of suppressing the flagellin-induced *NHO1* expression. The results demonstrated that flagellin-induced defenses play an important role in nonhost resistance. The effectors of secreted by TTSS of *Pst* DC 3000 act in the plant cell by suppressing the species level defenses (Li et al. 2005b).

The common assumption that bacteria can freely enter the plant through stomata on the leaf surface has been challenged by the findings on the interaction between *P. syringae* pv. *tomato* DC 3000 (*Pst* DC 3000) and *Arabidopsis* plants. Lipopolysaccharide (LPS)-triggered nitric oxide (NO) production and flagellin perception by its receptor FLS2 are required for resistance of *Arabidopsis* to *Pst* DC3000. The stomata in the *Arabidopsis* leaf epidermis have been shown to play a critical role in the innate immunity gates to actively prevent bacteria from entering the leaf tissue. Both plant bacteria *Pst* DC 3000 and human pathogeneic bacteria *E. coli* induced stomatal closure within the first hour of contact with plant tissue, suggesting that guard cells which form the stomata can sense conserved bacterial molecules such as PAMPs which have been shown to have the ability to stimulate innate immunity in plants and animals (Takeda et al. 2003). Both Flg22 and LPS were able to induce dramatic stomatal closure in the wild-type Co1-0 ecotype of *Arabidopsis.* The flg22 peptide did not induce stomatal closure in epidermal peels of *fls2* flagellin receptor mutant. However, LPS could induce stomatal closure in the mutant also. The results suggested that guard cell perception of flg22 required the FLS2 receptor which may be one among several receptors that may enable guard cells to sense multiple MAMPs displayed on the bacterial surface. Defense through stomatal closure has been shown to be an integral part of SA-regulated innate immune system (Melotto et al. 2006).

The differences in the stomatal responses of compatible and incompatible interactions were assessed. The responses of wild type Co1-0 plants to *Pst* DC3000

(compatible) and Pst DC3000/*avrRpt2* (incompatible) were studied. The avirulent strain *Pst* DC 3000/*avr Rpt* 2 induced stomatal closure within 1 h. The avirulent strain was less effective in reopening stomato compared with virulent strain at 3 h after incubation. The gene-for-gene resistance mediated by avr Rpt2/RPS2 appeared to have a positive effect on promoting stomatal closure. *Pst* DC 3000 produces coronatine, a polyketide toxin and it is an important virulence factor for infection of *Arabidopsis* and tomato plants (Brooks et al. 2004, 2005). COR was found to effectively inhibit abscisic acid (ABA)-induced stomatal closure. COR appears to counteract PAMP-induced stomatal closure downstream of ABA. Overcoming stomatal defense must be critically important in bacterial infection of plants. The results provide direct evidence that stomatal defense against bacterial infection is an important function of innate immunity in plants (Melotto et al. 2006).

The possible connections between innate immunity, race-specific and nonhost types of resistance responses were examined by using *Arabidopsis* cell cultures [Landsberg *erecta* (Ler)] and seedlings (Col-0) that were treated with flg22. The microarray analysis of transcripts showed that the majority of the *Flagellin Rapidly Elicited* (*FLARE*) genes were up-regulated and 80% of the encoded proteins had known or predicted function. The *FLARE* genes were functionally classified into signal transduction-related, signal perception-related, effector proteins or others groups. The mRNA for three auxin receptors were found to be negatively regulated by micro-RNA induced by flagellin perception. Enhancement of resistance to *P. syringae* infection occurred because of this down-regulation (Navarro et al. 2004, 2006). Many of the signal transduction-related genes encoded WRKY transcription factors are unique to plants and involved in regulating diverse plant functions including pathogen defense.

Localized or systemic defense responses depend on the activation of one or more of the signaling pathways SA, JA and ET or their derivatives. These pathways have been demonstrated to be associated with resistance to different types of pathogens. SA-dependent pathway mainly provides resistance to biotrophic pathogens. On the other hand, resistance to pathogens inducing necrosis is attributed to JA and ET pathways predominantly (Glazebrook 2005). Identification and utilizing key transcriptors in plant defense for engineering enhanced resistance to microbial pathogens may be expected to be an effective crop disease management strategy. The ET response factor (ERF) family is crucial, because its members function as a point of integration of JA and ET pathways. A large number of members (147) of the AP21 EREBP family of plant transcription factors is considered to be involved in diverse functions in *A. thaliana* (Feng et al. 2005; Nakano et al. 2006). The ERF or B subgroup of the AP2/EREB family, contain 65 *ERF* genes and all genes of the family are linked to disease resistance responses. The ERF genes are responsive to both JA and ET signaling pathways. Overexpression of several members of *ERF* genes led to increased expression of PR genes *PDF1.2, ChiB* and *Thi2.1* and increased resistance to a range of necrotrophic and biotrophic pathogens (McGrath et al. 2005).

Arabidopsis ERF genes, expression of which was specifically induced by *P. syringae* pv. *tomato* DC3000 (avrRpt2) infection with overlapping but distinct

induction kinetics, were identified. One of these genes, a transcriptional activator AtERF14 was induced at the same time as the ERF-target genes (*ChiB*, basic chitinase). The gain-and loss-of-function mutants were analyzed to determine the potential function of AtERF in regulating the plant defense response. Overexpression of AtERF 14 caused dramatic effects on both plant phenotype (severe growth retardation and loss of seed set) and defense expression. The *AtERF14* loss-of-function mutants exhibited impaired induction of defense genes following exogenous application of ET. In addition, susceptibility to *Fusarium oxysporum* was increased. The influence of AtERF14 on defense gene and *ERF* expression was studied using T-DNA insertion lines. The defense expression was not induced in the T-DNA insertion lines following alterations in two lines containing a T-DNA insertion into the coding sequence of AtERF14. However, the expression of both *PDF1.2* and *ChiB* was not affected due to treatment with ET, suggesting that AtERF14 is not only sufficient, but also essential for the activation of these genes. Furthermore, the expression of other ERF genes involved in defense and ET/JA responses such as ERF1 and AtERF2, depended on AtERF14 expression. A unique role for AtERF14 in regulating the plant defense response, such as loss of AtERF14 function on defense gene expression, pathogen resistance and regulation of the expression of other ERF genes is suggested by the results of this study (Oñate-Sánchez et al. 2007).

The *Cladosporium fulvum* effector protein Avr9, when extracellularly applied to tobacco cell culture, the group of genes called *Avr 9/Cf-9* were rapidly elicited (ACRE) as in the case of FLARE genes. Induction of *A. thaliana* (*At*) *ACRE* genes in response to Flg 22 was found to be similar in both suspension cells and seedlings, suggesting an overlap between the basal and gene-for-gene response to pathogens in *Arabidopsis* (Durrant et al. 2000). The study on the activities of bacterial effector proteins indicated that a nonpathogen is allowed to overcome some host defense system and they may function both as suppressors and inducers of plant resistance to the bacterial pathogen of tomato and bean (de Torres et al. 2006).

In tobacco, the early basal resistance (EBR), formerly designated ‘early induced resistance’ and late basal resistance (LBR) earlier known as ‘late induced resistance’ constitute a system that forms part of the innate immunity of plants. The EBR is triggered by general bacterial elicitors and EBR is considered to inhibit or retard expression of the type III secretion system (TTSS) of pathogenic bacteria and also to prevent nonpathogenic bacteria from colonizing the plant tissue. The rapidity of EBR development is crucial for halting pathogenesis and the cell wall may be a probable site, since bacteria cannot enter living plant cells. The nature of EBR-related proteins in the intercellular washing fluid (IWF) in which plant effector proteins were extracted, was investigated in tobacco-*P. syringae* pv. *tabaci* (*Ptab*) pathosystem. Several soluble proteins were detected in the IWF of tobacco leaf parenchyma that coincided with EBR and different environmental (light and temperature) conditions known to affect EBR. The EBR-related chitinases were expressed during HR. Two novel chitinases (EC 3.2.1.14) were trancriptionally induced before and during development of EBR. They were rapidly expressed in the apoplast and unlike PR-proteins, their expression was not stress-regulated. The EBR was induced by nonpathogenic, saprophytic and avirulent bacteria and chitinases accumulated

also under this condition. The outcome of bacterial infection may probably depend on the delicate balance between EBR, chitinases and the effectiveness of suppression of virulent *Ptab* (Ott et al. 2006).

Lipopolysaccharides (LPS) covering the cell surface of Gram-negative bacteria represent typical PAMP molecules capable of inducing defense-related responses including suppression of HR, the expression of defense genes and systemic resistance in plants. Although biological activities of LPS on dicot plants have been studied, no information regarding their function in monocot is yet available. Generation of ROS was induced by treatment with LPS in addition to induction of expression of PR-proteins (Gerber et al. 2004; Silipo et al. 2005). LPS treatment of *Arabidopsis* activated nitric oxide (NO) synthase and NO so evolved, has an important role in the activation of defense genes as well as resistance to pathogenic bacteria (Zeidler et al. 2004). Further, suppression of HR, a type of programmed cell death (PCD) associated with defense responses induced by avirulent bacteria has also been demonstrated (Erbs and Newman 2003). In an investigation on various bacterial LPS molecules including plant pathogens *P. syringae* pv. *syringae*, *Erwinia chrysanthemi*, *X. oryzae* pv. *oryzae* and *R. solanacearum* and nonpathogen were shown to induce defense responses such as ROS generation and defense gene expression in rice cells. Further global analysis of gene expression induced by LPS and chitin oligosaccharide (PAMPs) also showed a correlation between the gene responses induced by them. It is possible that there is a convergence of signaling cascades down-stream of their corresponding receptors. The defense responses induced by LPS in the rice cells have been demonstrated, for the first time, to be associated with PCD. However, PCD induction was not evident in cultured *A. thaliana* cells due to LPS treatment (Desaki et al. 2006).

Phospholipid signaling is considered to play an important role in plants, including responses to biotic and abiotic stresses. When challenged by an avirulent strain of *X. oryzae* pv. *oryzae* (*Xoo*), expression of phospholipase C (PLC) and phospholipase D (PLD) genes in rice were induced. The PLD localized in the plasma membrane, especially at the part adjacent to *Xoo* in incompatible interaction (Young et al. 1996). A race-specific elicitor AVR4, could induce a rapid accumulation of phosphatidic acid (PA) and diglycerol pyrophosphate (DGPP) in tobacco cells expressing the corresponding *Cf-4* resistance gene (de Jong et al. 2004). The synthetic diacylglycerol (DG) and PA were shown to rapidly induce transient generation of ROS (Yamaguchi et al. 2003). Induction of oxidative burst by exogenous application of PA to tobacco cells was demonstrated by de Jong et al. (2004). The effects of N-acetyl chito-oligosaccharide elicitor in rice cells were assessed. Biphasic generation of ROS induced by the elicitor in rice cells was associated with activation of PLC and PLD. The DG and PA also could induce the expression of elicitor-responsive genes in the absence of the elicitor. They themselves were able to induce biosynthesis of phytoalexins. However, they dramatically increased the elicitor induced phytoalexin accumulation. The results indicate the importance of phospholipid signaling in plant defense responses (Yamaguchi et al. 2005).

Interacellular recognition of both PAMP and Avr factors is primarily achieved by the nucleotide binding oligomerization domain (NOD) – protein family. Numerous

proteins of animals, plants and microbes origin are included in the NOD family (Inohara and Nunez 2003). Variations in the NOD family members may determine the levels of resistance to fungal, bacterial and viral pathogens demonstrating the essential role of the NOD-mediated innate immune response in plant and animal biology. The members of the toll-like receptor (TLR) family containing LRR in the extracellular domain and a TIR intracellular domain are involved in the recognition of PAMP in extracellular compartments or at the cell surface (Werling and Jungi 2003). The well-characterized example of plant host sensing and responding to PAMP at the cell surface is exemplified by the rice Xa21 receptor kinase. Recognition of bacterial strains of *Xoo* expressing AvrXa21 activity was due to Xa21 receptor kinase. The rice plants lacking Xa21 are susceptible to *Xoo.* In contrast, *Arabidopsis* plants lacking FLS2 did not exhibit increased susceptibility (Gómez-Gómez and Boller 2002). However, both FLS2 and Xa21 carry LRR in the presumed extracellular domain.

Four *Xoo* genes required for AvrXa21 activity, viz., *raxA*, *raxB raxC, raxST* were identified. These genes encode components of type I secretion system and sulfation system (da Silva et al. 2004). Two additional genes encoding a two-component regulatory system that are also required for AvrXa21 activity (*raxR* and *raxH*) were also identified (Burdman et al. 2004). Sequence analysis of three naturally occurring *Xoo* strains no longer recognized by Xa21 showed alterations in the *rax ST* and *rax* genes. The results indicated that bacterial type I secretion was essential for *Xa21*-mediated recognition and immunity. Further, type I secretion and modification of pathogen-associated molecules might play a significant role in triggering the innate immune response in rice. Characterization of the *rax* genes indicated that type I secretion (*raxA*, *raxB raxC*) and sulfation (*rax ST, rax P* and *rax Q*) are essential for Xa21 – mediated recognition of *Xoo* (Shen et al. 2002b; da Silva et al. 2004). In another study, two ORF encoding proteins with similarity to PR and HK of the OmpR/EnvZ class of bacterial two-component regulatory system were identified downstream of the *raxSTAB* genomic region. Inoculation experiments revealed that these ORFs were required for wild-type levels of Avr Xa21 activity and the genes were designated *raxR* and *raxH*. Inactivation of *raxR* or *raH* did not affect Avr Xa7 and AvrXa10 activities. The unique aspects of *Xoo-Xa21* system with respect to *avrR* gene interactions is brought to light by the fact that the Rax H/RaxR system affects Avr Xa 21, but not avrXa7 and AvrXa10 activities. The eight genes of *Xoo* required for AvrXa10 activity have been grouped into three classes: type I secretion, sulfur metabolism and two-component regulation. The results suggest that Avr Xa21 may represent an entirely new class of bacterial signaling molecules (Burdman et al. 2004).

Basal defense mechanisms are considered to be activated by surface-derived molecules referred to as pathogen-associated molecular patterns (PAMPs), whereas gene-for-gene based resistance is involved in recognition of the proteins encoded by *avr* genes. The response to lipopolysaccharides (LPS) and other PAMPs may be regarded as an expression of a basal resistance which has to be overcome by a microbe to become a successful pathogen. The more specific gene-for-gene interactions that control varietal resistance and HR are superimposed on this PAMP-

mediated basal resistance. An integrated comparative proteomics and transcriptomics approach was applied to dissect the signaling and response pathways operating during basal and gene-for-gene mediated resistance in the model pathosystem *Pst* DC 3000 and *A. thaliana*. Protein changes characteristic of the establishment of basal resistance and R-gene mediated resistance was investigated by comparing responses to *Pst* DC 3000, a *hrp* mutant and DC 3000 expressing *avrRpm1* respectively. The changes in two antioxidant enzyme group viz., the glutathione-S-transferases (GSTs) and peroxiredoxins (Prx) were studied. All forms of GST proteins (F2, F6, F7) increased following inoculation with bacteria. GSTF8 showed more dynamic responses to pathogen challenge, the corresponding transcript was significantly up-regulated by 2 h after inoculation and the protein exhibited post-translational modifications (PTMs) specific to incompatible interaction. The results suggested that bacterial infection generally induced Prxs and the antioxidant GSTs (Jones et al. 2004).

Resistance induced in tissues far away from the initial infection sites by SAR, following gene-for-gene recognition between plant resistance proteins and pathogen effectors is yet to be thoroughly understood. Although SA participates in the local and systemic response, SAR does not require long-distance translocation of SA. However, SAR depends on the accumulation of SA in distal leaves where it induces a change in cellular redox triggering in the reduction of oligomeric disulfide bound NPR1′ (nonexpressor PR genes) a central regulator of SAR. The systemically responding leaves rapidly activate a SAR transcriptional signature with strong similarity to local basal defense. Evidence suggesting a central role for jasmonates in systemic defense has been obtained, possibly acting as the initiating signal for classic SAR. Jasmonic acid (JA), but not SA, rapidly accumulated in phloem exudates of leaves challenged with an avirulent strain of *P. syringae.* Transcripts associated with jasmonate biosynthesis were up-regulated in systemically responding leaves within 4 h and JA increased transiently. SAR was abolished in mutants defective in jasmonate synthesis or response. The results indicated that jasmonate signaling seemed to mediate the long-distance information transmission (Truman et al. 2007).

The phytohormones SA, JA and ET are known to participate in regulating defense responses in plants. SA is predominantly associated with resistance against biotrophic and hemibiotrophic pathogens and establishment of SAR (Grant and Lamb 2006). In contrast, JA and ET dependent defense mechanisms are involved in resistance to necrotrophic pathogens, suggesting that the signaling network engaged by the host is dependent upon the nature of the pathogen and its mode of pathogenicity (Glazebrook 2005). The role of abscisic acid (ABA) in plant responses to infection by bacterial pathogens was investigated. ABA-deficient mutants exhibited reduced susceptibility to virulent isolates of *P. syringae* pv. *tomato* (*Pst*) DC 3000 in tomato (Thaler and Bostock 2004), suggesting a negative role for ABA in disease resistance. A later study showed that ABA signaling pathway was found to be a major target for effectors secreted by *Pst* DC 3000. Microarray data identified a prominent group of effector-induced genes that were associated with ABA biosynthesis and also responses to this plant hormone. Conditional expression of a single bacterial effector Avr PtoB could enhance bacterial growth, elevate ABA levels and

suppress PAMP-responsive genes. Among the genes upregulated by effector delivery *NCED3,* encoding a key enzyme of ABA biosynthesis and the ABA insensitive 1 (*ABI1*) clade of genes encoding protein phosphatases type 2C (*PP2Cs*) involved in the regulation of ABA signaling were the important ones. Modification of PP2C expression resulting in ABA in sensitivity or hypersensitivity led to restriction or enhanced multiplication of bacteria respectively. Levels of ABA increased rapidly during bacterial colonization of *A. thaliana* tissues. Expression of AvrPtoB *in planta* modified host ABA signaling (de Torres-Zabala et al. 2007).

The NPR1 gene (also designated NIM1 and SAI1) is the key regulator of SA-mediated SAR in *Arabidopsis thaliana*. SA-induced PR genes (SAR) and some *R* gene mediated resistance were not expressed in *npr1* mutants. But overexpression of *NPR1* enhanced resistance to diverse pathogens in a dose-dependent manner in *Arabidopsis* (Friedrich et al. 2001). *NPR1* has been shown to coordinately induce secretion-related genes required for PR protein secretion during SAR (Wang et al. 2005). The *NPR1* is functionally conserved in diverse plant species including rice. Studies were taken up to have an understanding of NPR1-mediated resistance in rice and to develop a practical strategy for enhancing disease resistance in the model crop rice. SA may play a role as defense signal in rice also, as the transgenic rice expressing the *nahG* gene, encoding a salicylate hydroxylate, failed to accumulate SA and exhibited higher level of susceptibility to rice blast disease (Yang et al. 2004). Furthermore, overexpression of *NPR1* and the rice *NH1* gene (NPR1 homolog 1) also increased disease resistance in transgenic rice (Chern et al. 2001, 2005).

The rice *NPR1* homologous genes were isolated and transgenic rice overexpressing these genes were generated. The rice homologous gene *NH1* was shown to be the rice *NPR1* ortholog (termed hereafter *OsNPR1*) through the complementation of the *Arabidopsis npr1-1* mutant. Overexpression of OsNPR1 conferred high levels of resistance to rice bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In contrast, *OsNPR1* knockout/knock down plants displayed higher susceptibility to *Xoo*. The wild-type OsNPR1 protein (in GFP fusion) was located in the cytoplasm and it was able to move into the nucleus after redox change. Mutations in its conserved cysteine residues resulted in the constitutive localization of OsNPR1 (2CA)-GFP in the nucleus and abolition of herbivore hypersensitivity in transgenic rice. Different sub-cellular localizations of OsNPR1 antagonistically regulated SA-and jasmonic acid (JA)-responsive genes, but not SA and JA levels. This condition indicates that OsNPR1 might mediate antagonistic cross-talk between the SA and JA dependent pathways in rice. *PR* genes appear to be differently expressed in *Arabidopsis* and rice. Overexpression of *OsNPR1* did not increase resistance to blast disease. Probably SAR-like disease resistance was activated against bacterial blight disease through SA signaling which might be less dependent on the elevation of SA levels in rice cells. This investigation highlights the possibility of engineering rice with a broad spectrum resistance to *Xoo* using the site-mutated *OsNPR1* gene (Yuan et al. 2007).

The plant defense responses are induced by a complex signaling network interconnected by cross-talk to networks regulating other plant functions. The crucial

mediators of plant resistance are the phytohormones salicylic acid (SA) and jasmonic acid (JA). In addition to SA and JA, the major transcriptional reprogramming associated with the plant defense response requires also the action of diverse transcription factors (Chen and Chen 2002; Eulgem 2005). The WRKY class of transcription regulators seem to play a major role in the regulation of plant defense responses. The conserved elements of the domain are essential for the high binding affinity of WRKY proteins to the consequence designated the W-box (Zhang and Wang 2005). In *Arabidopsis*, 74 WRKY proteins classified into three groups (I, II and III) have been identified. Since majority of WRKY genes of *Arabidopsis* are upregulated in defense responses or after treatment with defense-inducing elicitors or hormones, they are considered as crucial regulators of the pathogen-induced active defense response. An expression profiling study showed that 49 of 72 WRKY genes in *Arabidopsis* were induced following application of SA or *P. syringae* pv. *tomato* (*Pst*) (Dong et al. 2003). The importance of WRKY70 for *Erysiphe cichoracearum* resistance (Li et al. 2006) and homologous genes WRKY18, WRKY40, and WRKY60 in resistance to *Pst* and *Botrytis cinerea* (Xu et al. 2006) has been established. The role of subfamily II of WRKY transcription factors in the regulation of basal resistance to *Pst* was studied. The virulent and avirulent strains of *Pst*, on inoculation, induced the expression of four members of this subfamily. Loss of function of WRKY11 enhanced resistance toward both avirulent and virulent strains. Furthermore, the double mutants of *Arabidopsis (wrky11 wrky17*) exhibited further increase in the level of resistance to *Pst*, indicating that WRKY11 and WRKY17 acted as negative regulators of basal resistance to *Pst.* In response to challenge by *Pst*, these two transcription factors modulated transcriptional changes and they acted either specifically or in a partially redundant manner, depending on the target gene. The results showed that both WRKY transcription factors are involved in the regulation of *Pst*-induced jasmonic-acid dependent responses (Journot-Catalino et al. 2006).

Plant bacterial pathogens produce several type III effectors (Volume 2, Chapter 2) which are designated avirulence (Avr) factors involved in elicitation of resistance to pathogen strain/species concerned. In the presence of disease resitance (*R*) genes in specific plant genotypes Avr factors trigger potent gene-for-gene resistance and HR – localized programmed cell death (PCD) (Dangl and Jones, 2001). One of the TTSS effectors of *P. syringae* triggers disease resistance genes in tomato plants carrying the corresponding *R* gene *Pto* that encodes a serine/theronine kinase (Pedley and Martin 2002). The *Pto* gene can also recognize another TTSS effector AvrPtoB which has little sequence similarity to Avr Pto which enhances pathogen growth and virulence in the absence of Pto. The functions of Avr effectors may be related to either triggering resistance or enhancing pathogen virulence (Shan et al. 2000). The *avrPto* and *avrPtoB* are the only avirulence genes in DC 3000 that elicit Pto-mediated defense responses in tomato. The avr PtoB deletion mutant of *Pst* DC 3000 retained Pto-specific avirulence on tomato. The double mutant DC 3000 Delta avr Pto Delta avr PtoB caused less severe disease symptoms than single mutants and grew more slowly than the single mutants on susceptible leaves. The results indicate that AvrPto and AvrPtoB have phenotypically redundant avirulence activity on Pto-expressing tomato

and additive virulence activities on susceptible tomato plants (Lin and Martin 2005). In a later investigation, a cell-based genetic screen of virulence factors of *Pst* DC 3000 was performed. AvrPto and Avr PtoB were identified as suppressors of early defense gene transcription and MAPK signaling. AvrPto and AvrPtoB were shown to intercept multiple MAMP-mediated signaling (He et al. 2006a).

Pseudomonas syringae pv. tomato (*Pst*) has the unrelated effector proteins AvrPto and Avr PtoB that can trigger disease resistance in tomato cultivars containing the *Pto* gene. Pto, a protein kinase interacts directly with each effector through overlapping surface areas (Pedley and Martin 2002; Wu et al. 2004). The NBARC-LRR (nucleotide binding domain shared by Apaf-1, certain R gene products and CED-4 fused to C-terminal leucine-rich repeats) protein Prf is required for Pto to function and the corresponding genes *Pto* and *Prf* are clustered at a single genomic locus with four *Pto* homologs (Chang et al. 2002). Immunity in tomato to *Pst* expressing the effector proteins AvrPto and AvrPtoB was dependent on Pto kinase and the NBARC-LRR protein Prf. Pto had a direct role in effector recognition within the host cytoplasm and the role of Prf is not clearly understood. Pto and Prf were found to be coincident in the signal transduction pathway controlling ligand-independent signaling. The results suggested that the role of Pto was confined to the regulation of Prf and the bacterial effectors might have evolved to target this coregulating molecular switch (Mucyn et al. 2006).

In *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) the AvrPtoB type III effector elicits immunity-associated programmed cell death (PCD) when expressed in tomato plants carrying the Pto resistance protein. In contrast, in the absence of Pto, the same AvrPtoB functions as suppressor of PCD and immunity in tomato. The PCD eliciting and cell death suppressor (CDS) activities of Avr PtoB have been mapped to distinct regions of the protein. AvrPtoB was shown to be a modular protein with cell death eliciting and suppressing activity in the N-and C-termini, respectively. Deletion of as few as 44 amino acids from the C-terminus caused a loss of CDS activity. An AvrPtoB mutant lacking CDS activity resulted in Pto-independent PCD in both tomato and *N. benthamiana*. This gain of PCD was considered to be due to suppression of PCD triggered by a normally hidden *R* gene designated *Rsb* (*R*esistance *s*uppressed *b*y the *avrPtoB* C-terminus). A fragment of AvrPtoB containing amino acids 1-308 was enough to elicit *Rsb*-mediated HR (Abramovitch and Martin 2005). Avr Rpt2 an effector protein from *Pst* DC 3000 functions as an aviruelnce factor that activates resistance in *A. thaliana* lines expressing the resistance genes *RPS2*. On the other hand, AvrRpt2 can also increase pathogen fitness by supporting the ability of *Pst* DC 3000 to multiply and induce disease symptoms on susceptible *A. thaliana* lines lacking functional *RPS2*. The activation of RPS2 appears to depend on the AvrRpt2-induced elimination of *A. thaliana* RIN4 proteins (Lim and Kunkel 2004).

Caspases are components of the cell death machinery universally involved in animal apoptosis. They are a family of separate-specific cysteine proteases that have a role in initiating and executing PCD (Shi 2002). In plants evidence for the existence of caspase-like activities has been observed and their requirement for PCD has been indicated (del Pozo and Lam 1998; Lam 2004). The vacuolar processing enzymes

(VPE) α,β,γ, and δ are *Arabidopsis* legumains essential for the processing of vacuolar proteins (Shimada et al. 2003). VPEγ is the isoform of most highly expressed in vegetative tissues where it plays a role in protein degradation during senescence, a form of PCD in plants (Rojo et al. 2003). VPEγ was shown to bind in vivo to a general caspase inhibitor and to caspase-1-specific inhibitors which block VPEγ activity. In *vpe*γ mutants, a cysteine protease inhibitor cystatin, accumulated to 20 fold higher levels. Infection of *A. thaliana* with an avirulent strain of *P. syringae* pv. *tomato* (*Pst*) resulted in an increase in caspase-1 activity and such an increase was suppressed partially in *vpeγ* mutants. Plants overexpressing VPEγ exhibited more intense ion leakage during infection with *Pst* suggesting that VPEγ expression following infection with *Botrytis cinerea* and *Turnip mosaic virus* was also observed. Knockout of *VPE*γ led to higher level of susceptibility to bacterial, fungal and viral pathogens included in this investigation (Rojo et al. 2004).

Resistance to *Pseudomonas syringae* pv. *maculicola* 1 (*RPM1*) encodes a CC-NB-LRR protein that confers resistance against *P. syringae* either of two sequence unrelated type III effectors AvrB and Avr Rpm1. RPM1-interacting protein (RIN4) was found to be associated with both RPM1 and resistance to *P. syringae* 2 (RPS2) disease resistance proteins. RIN4 is plasma membrane-localized and it is required for RPM1-mediated disease resistance being responsible for RPM1 accumulation before infection. Upon infection with *P. syringae* expressing either AvrB or Avr Rpm1, RIN4 was phosphorylated (Lee et al. 2004). RPS2 conferred resistance to *P. syringae* expressing the type III effector AvrRpt 2 which is a putative Cys protease (Axtell et al. 2003). AvrRpt2 causes post-transcriptional disappearance of RIN4 which may be delayed by over expression of RIN4 resulting in inhibition of RPS2 activation. Further RIN4 also negatively regulates inappropriate activation of both RPM1 and RPS2. Inappropriate activation of RPS2 was nonspecific disease resistance 1 (NDR1) independent. In contrast, NDR1 was required during AvrRpt2-dependent RPS2 activation. RIN4 might act either cooperatively, downstream or independently of NDR1 to negatively regulate RPS2 in the absence of the bacterial pathogen (Belkhadir et al. 2004).

Bacterial effector proteins secreted by TTSS were discovered as avirulence determinants and they were directly or indirectly recognized by their cognate resistance (*R*)-gene products. The *Pseudomonas* effector *avrRpm1* was induced within an hour of leaf inoculation (Thwaites et al. 2004). The plants do not have enough *R* genes to intercept all potential avirulence determinants secreted by bacterial pathogen. Hence, *R* proteins guard the effector targets either directly or by sensing modification of the target (Mackey et al. 2002). The defense reaction initiated by R proteins commonly leads to a form of PCD known as HR and subsequent pathogen restriction. The changes in the proteome of *A. thaliana* leaves during responses to challenge by *Pst* DC 3000 were analyzed. Protein changes characteristic of the establishment of disease, basal resistance, and resistance gene-mediated resistance were assessed by comparing responses to *Pst* DC3000, a *hrp* mutant and *Pst* DC3000 expressing *avrRpm1* respectively. The proteins showing remarkable changes after pathogen challenge represented two main functional groups *viz.,* defense-related antioxidants and metabolic enzymes. Changes to primary metabolism and an-

tioxidant enzymes involved in basal defense were shown to be altered following introduction of bacterial effectors. Proteins specific to the establishment of disease were identified. Components of photosystem II(PSII), mitochondrial permeability transition (MPT) and cytoplasmic antioxidant enzymes were modified during *R* gene-mediated HR (Jones et al. 2006).

Endogenous small interfering RNAs (siRNAs) and microRNAs (miRNAs) have been demonstrated to be important regulators of eukaryotic gene expression by guiding mRNA cleavage, translational inhibition or chromatin modification (Sontheimer and Carthew 2005). RNA interference mediated by siRNAs is a conserved regulatory process that has evolved as a natural antiviral defense mechanism in plants and animals. Contribution of a miRNA to basal defence against *P. syringae* by regulating auxin signaling was demonstrated by Navarro et al. (2006). An endogenous siRNA, natural nat-siRNA ATGB2 was specifically induced by *Pst* (avrRpt2). This nat-siRNA was synthesized by a unique biogenesis pathway that required *DCL1, HYL1, HEN1, RDR6, SGS3* and RNA polymerase IVa. This nat-si RNA was demonstrated to be produced following induction of the sense transcript ATG B2, and host resistance gene RPS2 and its resistance signaling components including NDR1. The specific induction of nat-siRNA ATGB2 resulted in the silencing of antisense gene PPRL which is likely to be a negative regulator of RPS2 signaling pathway. Silencing of PPRL by nat-si RNA ATGB2 was found to have a positive role in the development of disease resistance. This investigation appears to be the first in establishing a role for endogenous siRNA in bacterial disease resistance in *Arabidopsis.* The results indicate that endogenous si-RNA-mediated gene silencing may serve as an important mechanism for gene expression reprogramming in plant defense responses (Katiyar-Agarwal et al. 2006).

The AvrBs3-like effector proteins from different of *Xanthomonas* spp. are numerous and highly similar. They together with their corresponding R proteins form a unique biological resource to dissect the molecular basis of recognition specificity. Recognition of AvrBs3 and AvrBs4, two nearly identical bacterial effector proteins from *X. campestris* pv. *vesicatoria* (*Xcv*) that are specifically detected by cognate pepper Bs3 and tomato Bs4 respectively, was studied. These two proteins belong to a large family of highly similar, nuclear targeted Avr proteins. The structural feature of AvrBs3 and homologous proteins shows the presence of repeat domain made up of 5.5–25.5 nearly identical tandemly arranged copies of a 34 amino acid (aa) repeat unit in common (Lahaye and Bonas 2001). Brg 11 from *R. solanacearum* (Cunnac et al. 2004) and Hax2 from *X. campestris* pv. *armoraciae* (Kay et al. 2005) show structural variations. The appearent inter-connection between Avr and virulence functions of many *avrBs3*-like genes was indicated by mutational analysis (Lahaye and Bonas 2001). The functionality of Bs3, Xa7, and Xa10-encoded proteins relies collectively on the presence of functional NLSs and ADs in their cognate Avr proteins, suggesting that both employ similar perception mechanisms (Schornack et al. 2006).

The virulence and Avr activities of some avrBs3 have been shown to be separable as in *X. oryzae* pv. *oryzae* (*Xoo*) *avrXa7* gene. The avrBs3-like *avrXa7* gene triggered a defense response in rice lines containing the corresponding *Xa7* disease resistance gene. In contrast, *avrXa7* enhanced the aggressiveness of *Xoo* in

rice lines that lack the *Xa7* resistance gene (Bai et al. 2000; Porter et al. 2003; Yang 2005). Likewise, AvrBs4 protein of *Xcv* triggered a defense response in tomato genotypes expressing the matching Bs4 resistance. The AvrBs4 was shown to be a virulence factor that promoted pathogen development in planta (Ballvora et al. 2001; Wichmann and Bergelson 2004). Although AvrBs3 and AvrBs4 are highly similar (96.6%), the pepper Bs3 and the tomato Bs4 protein are able to mediate specific recognition of avrBs3-and avrBs4-expressing *Xanthomonas* spp. (Ballvora et al. 2001). The pepper *Bs3* gene retained its recognition specificity even if *avrBs4* was expressed in planta from a strong 35S promoter. But the recognition specificity of *Bs4* was retained, if in planta expression of the *avrBs3*-like gene was driven by the weak promoter instead of the strong 35S promoter (Schornack et al. 2004, 2005). The rice *R* gene *Xa27* mediating recognition of *Xoo* isolates that express avrXa27 is a new member of the avrBs3 family (Gu et al. 2005). Xa27 functionally resembles pepper Bs3 and many other *R* genes that mediate recognition of AvrBs3-like proteins in an NLS-and AD-dependent manner. Xa27 showed no apparent sequence homology to proteins from other organisms other than rice. Strangely, the resistant and susceptible parental rice lines of mapping population encode identical Xa27 proteins. Expression of the *Xa27* allele occurred only when the rice plant was challenged by *Xoo* harboring avrXa27, but not by isogenic strains lacking avrXa27 (Schornack et al. 2006).

The bacterial avirulence gene function is dependent on interactions with HR and pathogenicity (*hrp*) genes. The *avr* gene products are believed to be translocated into the cytoplasm by the *hrp*-encoded protein translocation complex. The *hrp*-encoded protein secretion apparatus also translocates harpins which are glycine-rich proteins produced when *hrp* genes are expressed. Harpins are produced in addition to Avr determinants. Harpins may not be the primary elicitors of a defense response, at least in *P. syringae* strains (Alfano et al. 1997). The elicitor activity of harpins is entirely dependent on other genes such as *hrmA* from *P. syringae* indicating that avirulence genes are indispensable for elicitation of a defense response (Alfano et al. 1997). The presence of a novel plant protein HR-assisting protein (HRAP) in sweet pepper that could intensify the $harpin_{pss}$ – mediated HR in $harpin_{pss}$ – insensitive plants was detected. The *hrap* mRNA accumulated preferentially during the incompatible interaction of sweet pepper leaves with *P. syringae* pv. *syringae* (*Pss*). The sweet pepper leaves readily expressed the $harpin_{pss}$ – mediated HR when the *hrap* gene transcription reached high levels. All organs of sweet pepper, except the fruit, expressed two different forms of HRAP. As the *hrap* gene is widely distributed in many plants species, the interaction between HRAP and $harpin_{pss}$ may help to understand the mechanisms of interaction between plants and bacterial pathogens (Chen et al. 2000).

The virulence determinants harpin (Hrp N) and polygalacturonase (Peh A) from hrp-positive strain of the soft rot pathogen *Erwinia carotovora* subsp. *carotovora* (*Ecc*) were employed as tools to elucidate plant responses. In the nonhost *Arabidopsis*, establishment of resistance by HrpN was accompanied by the expression of salicylic acid (SA)-dependent, but also jasmonate/ethylene (JA/ET)-dependent, marker genes *PR1* and *PDF1.2*, respectively. Apparently both SA-dependent and

JA/ET-dependent pathways were activated. These two elicitors, HrpN and PehA, also cooperated in triggering increased production of superoxide and lesion formation (Kariola et al. 2003). Evidence suggesting that resistance of tomato and pepper to T3 strains of *Xanthomonas campestris* pv. *campestris* (*Xcc)* was specified by a plant-inducible avirulence (*avr*) gene was presented by Astua-Monge et al. (2000). HR in certain tomato and pepper genotypes was elicited by tomato race 3 (T3) of *Xcc*. The *avrXv3* gene was found to be plant inducible and controlled by the *hrp* regulating system. The *Agrobacterium*-mediated transient expression confirmed that AvrX3 had a direct role in eliciting HR in tomato (NIL 216), providing support to the view that Avr proteins are located inside the host plant cell to trigger HR (Astua-Monge et al. 2000).

The bacterial proteins harpins, avirulence proteins and pectic enzymes significantly affect the plant pathogen interactions including HR and disease progression. Pectate lyases affect the cell wall structure resulting in tissue maceration and cell death. Their involvement in induction of HR was investigated in *Xanthomonas axonopodis* pv. *glycines* (*Xag*) strains. By mutational analysis, several genes on pathogenicity island in *Xag* strain 8ra were shown to be essential for HR induction. Two of the genes *hrpG* and *hrpX* were found to be involved in the regulation of several of the *hrp*, *hrc* and *hpa* (HR-associated) genes. Infiltration of leaves with 8ra cells resulted in an HR on pepper and tomato plants, but not on tobacco. However, transformation of 8ra with a multicopy plasmid expressing *hrpG* and *hrpX*, resulted in restoration of the ability to induce HR on tobacco (Kim et al. 2004b). In a later study, transposon insertional mutant KU-P-M670 of *Xag* generated from wild-type strain KU-P-34017 lost the ability to induce HR on tobacco and pepper, but retained HR induction ability on cucumber, sesame and tomato. Multiple mechanisms and elicitors of the HR may be present in *Xag* and the HR on tomato may be induced in a hrp-dependent manner. The ability of the strain KU-P-34017 to induce HR on tobacco was found to be associated with the presence of a putative gene, *xagP* that conferred the expression of pectolytic activity. The wild-type strain CM9-015 did not carry *XagP* and did not induce HR on tobacco. Complementation of the mutant KU-P-M670 with *XagP* restored the ability to induce HR on tobacco and pepper. *Xag* appears to be a highly heterogenous species with varying ability for pathogenicity and HR on different plant species (Kaewnum et al. 2006). This report seems to be the first one indicating a role for functional pectate lyase in induction of HR on plants.

Bacterial harpin proteins form an important group of TTSS effectors that elicit multiple plant responses during plant-bacteria interactions. The beneficial effects of harpins on plants have been studied in several pathogen-host combinations. In addition to enhancing host defense response, the harpins stimulate plant growth. The harpin proteins contain repeats of a glycine-rich motif (GRM), as found in $HrpN_{Ea}$ of *Erwinia amylovora* (*Ea*) and $HrpZ_{pss}$ of *Pseudomonas syringae* pv. *syringae* (*Pss*) (Wei et al. 1992; He et al. 1993).The harpin genes in *Xanthomonas* spp. such as $hpaG_{xoo}$ in *X. oryzae* pv. *oryzae* (*Xoo*) and $hpaG_{xooc}$ in *X. oryzae* pv. *oryzicola* have been identified by targeting *hpa* (*hrp*-associated) loci (Wen and Wang 2001; Li et al. 2004). Proteins of harpins induced HR, hypersensitive cell death (HCD)

and systemic acquired resistance (SAR). Two mutant versions of $HpaG_{xooc}$ were shown to be more effective than the wild-type protein in inducing plant defense and growth. The mutant C47T was similar to $HpaG_{xooc}$ in eliciting HCD. However, it induced greater levels of defense and growth. The results indicate that harpins may act similarly by applying intramolecular regulation mechanism to different functions. Several regions in $HpaG_{xoo}$ (Peng et al. 2004) and $HpaG_{xooc}$ (Liu et al. 2006) when isolated from the proteins were shown to be more active than intact molecules to differentially elicit HCD, plant growth enhancement (PGE) and pathogen defense. The intact proteins may impair full effects of the functional regions of harpins. The results of this investigation have potential relevance for agricultural production by enhancing plant disease resistance and plant growth by application of selected regions of harpins (Liu et al. 2006).

Erwinia amylovora (*Ea*) causing the fire blight diseases, elicits a hypersensitive response (HR) in nonhost plants such as *A. thaliana.* The gene cluster *hrp* is necessary for pathogenicity on host plants and HR elicitation on nonhost plants. Four proteins of *Ea* secreted via Hrp pathway are Dsp A/E, HrpW, HrPA and harpin N_{Ea}. When purified $HrpN_{Ea}$ was infiltrated into tobacco leaves, HR was induced. The gene encoding $HrpN_{Ea}$ named hrpN is located on the bacterial chromosome (Barny et al. 1999). Harpin treatment in tobacco caused rapid inhibition of ATP synthesis, suggesting alterations of mitochondrial functions (Xie and chen 2000). $HrpN_{Ea}$ also induced SAR in *A. thaliana* against *Peronospora parasitica* and *P. syringae* pv. *tomato*, accompanied by induction of SAR genes PR-1 and PR-2 (Wei et al. 1998).

In order to create novel mechanisms for fire blight resistance in pear, transgenic pears expressing the elicitor HrpNEa under the control of the constitutive promoter CaMV35S. No apparent damage due to transient expression of $hrpN_{ea}$ could be seen in pear cells. Hence, effects of stable constitutive expression of $hrpN_{ea}$ in 17 transgenic clones of the highly susceptible cv. 'Pase Crassane' were investigated. Significant reduction in susceptibility to fire blight was observed when inoculated with *Ea* under in vitro conditions. There was positive correlation between the degree of expression of the transgene $hrpN_{ea}$ and reduction in susceptibility to *Ea*, indicating that ectopic expression of a bacterial elicitor-like $HrpN_{ea}$ can be an effective strategy for improving resistance of pear cultivars to fire blight disease (Malony et al. 2005). The HrpN(ea) harpin from *Ea* induced cell death and H_2O_2 in the nonhost *A. thaliana,* but did not induce such responses in host MM106 apple cells. Further, HrpN(ea) also induced an increase in anion current in host MM106 apple cells. In contrast, a decerease of anion current was noted in nonhost cells which was responsible for induction of cell death in nonhost *A. thaliana,* suggesting operation of different signaling pathways in compatible and incompatible interactions (Reboutier et al. 2007).

Phytoalexins, low molecular weight compounds, accumulate in the plants in response to infection or abiotic elicitors (Kuć 1995). In cotton, cadinane-type sesquiterpenes including cadalene derivatives (7-hydroxylated cadinanes) are produced mostly as phytoalexins in response to stress (Bell 1986). Cotton leaves infected by incompatible races of *X. campestris* pv. *malvacearum* (*Xcm)* (causing

bacterial blight disease) induced the cadalene derivatives as the major phytoalexins as part of the HR which forms an important component of resistance to bacterial blight (Pierce et al. 1996). The enzyme (+) – δ-cadinene synthase (CDNS) catalyzes the first step in the biosynthesis of cadinene-type sesquiterpenes, such as gossypol which provide constitutive and inducible protection against diseases. The CDNS genes belong to a large multigene family, of which five genomic clones, including three pseudogenes and one gene respresenting another subfamily of CDNS were examined. Infection by *Xcm* induced CDNS expression in cotton. Constructs for the constitutive or seed-specific antisense suppression of *cdn1-C4* were introduced into cotton by *Agrobacterium*-mediated transformation. The induction of CDNS mRNA and protein in response to bacterial blight infection of cotyledons was completely blocked in the constitutive antisense plants. The results suggest that *cdn1-C*4 may be specifically involved in response to bacterial blight infection (Townsend et al. 2005).

3.3 Viral Diseases

3.3.1 Genetic Basis of Resistance

Plant viruses are relatively simple genetic entities. Yet the mechanisms of induction of disease symptoms and the mechanism by which plants resist the adverse effects of viruses are largely unknown. The responses of plants to viral infections have been designated in different ways and the terms representing the phenomenon of resistance to viruses have been found to overlap or some being inappropriate. Immunity to virus infection is observed in certain plant species (nonhosts) in which no detectable virus replication may occur either in the inoculated protoplasts or cells of intact plants; consequently no virus progeny is produced. In some apparently immune plants, 'subliminal' infection of a single or a few cells which are inoculated directly may be detected (Fraser 1990; Matthews 1991). The term infectible was suggested by Cooper and Jones (1983) to represent plant response which is opposite of immunity. Occasionally such type of interaction may be seen in cultivar resistance, as in the case of resistance offered by *Tm2* and *Tm2*^{2} genes against *Tomato mosaic virus* in tomatoes (Nishiguchi and Motoyoshi 1987). The resistance conferred by a gene against viral infection of a cultivar is termed cultivar resistance which may become ineffective, when a virulent strain is formed by either mutation or selection from a mixture. Infection, in resistant plants, may be limited by a host response to a zone of cells around the initially infected cell, usually by producing visible necrotic local lesions. This is known as the hypersensitive response (HR). The plants exhibiting such HR are field resistant. Tolerance is the resistance to symptom development rather than to virus replication and this type of resistance is not seen in infection by fungal or bacterial pathogens. When plants react with severe disease symptom, the response is considered as sensitive. The plants exhibiting tol-

erance and sensitive response are considered to be susceptible, as the virus becomes systemic and replicates freely. When the virus becomes systemic, the response is termed susceptibility (Matthews 1991).

The successful deployment of the desired gene(s) into crop plant depends more upon the identification of a positive phenotype, dissection of the phenotype resulting in the identification of genetic markers for marker-assisted selective (MAS) breeding and an understanding of how the novel resistance will perform in different genetic backgrounds and under pathogen pressure in the field. As in the case of fungal and bacterial pathogens, the major virus disease resistance genes belonging to the NBS-LRR class confer complete resistance (qualitative), but they are not always associated with cell death and/or tissue necrosis. For example, the *Rx* resistance against *Potato virus X* (PVX, *Potexvirus*) which confers extreme resistance that inhibitis virus replication without HR being apparent. Similar responses have been reported for *Sw5* in tomato and *Rsv1* in soybean (Bendahmane et al. 1999; Bromonschenkel et al. 2000; Hayes et al. 2004). The virus *R* genes appear to have a wider specificity than that was expected as in the case of *Sw5* conferring resistance to several tospoviruses (Bromonschenkel et al. 2000). Since viruses depend mostly on the vectors for dissemination, genes that resist infestations with the vectors and/or block virus transmission may be able to provide additional scope for genetic resistance (Maule et al. 2007).

Development of a reliable method of screening cultivars and genotypes to assess their level of resistance to the virus disease in question is a prerequesite for production of cultivars resistant to the target virus. Several parameters such as intensity of disease symptoms, growth abnormality and extent of yield reduction have been considered. The effects of virus infection may be influenced by environment and plant nutrition and disease pressure. A disease rating scale for *Tomato yellow leaf curl virus* (TYLCV) disease was developed based on disease symptom intensity, plant height and virus DNA content in the inoculated plants. The scale comprised of seven different homozygous tomato genotypes that exhibited different levels of TYLCV resistance from STY-1 representing high susceptibility to STY-7 representing high resistance, showing little disease symptoms. TYLCV DNA accumulation in the uppermost leaf of infected plants was estimated by dot-blot hybridization. The amount of viral ss-DNA in each spot was quantitated and the background level was subtracted from each measurement. The amount of TYLCV DNA in samples was calculated using a standard graph based on purified virus preparation and expressed as the percentage of DNA in the susceptible STY-1 plants which was equated to 100%. The STY-1 plants had the highest concentration of viral DNA, less in moderately resistant genotypes (STY2 and STY3) and very low levels of viral DNA were present in the highly resistant plants (STY-4 toSTY-7) (Table 3.3) (Lepidot et al. 2006)

The resistance to virus diseases has a unique aspect. Resistance to the virus and resistance to the disease symptoms have to be differentiated. Resistance to the virus ultimately results in resistance to disease. However, necrosis as part of resistance response may prove to be lethal as in *N* gene resistance to *Tobacco mosaic virus* (TMV) (Dijikstra et al. 1977) or the I gene resistance to *Bean common mosaic virus* in bean (*Phaseolus vulgaris*) (Collmer et al. 2000). The virus is able to become

Table 3.3 Levels of resistance of tomato differential genotypes to TYLCV as determined by different parameters

Genotypes	Symptom rating score (0–4)	Height of plants (%)*	TYLCV DNA (%)**
STY – 1	4.0	47 a	100.0 a
STY – 2	3.4	48 a	45.7 b
STY – 3	3.2	60 a	34.2 b
STY – 4	2.0	87 c	7.7 c
STY – 5	1.3	73 b	3.1 c
STY – 6	0.8	62 a	6.9 e
STY – 7	0.2	74 b	5.5 c

* Percentage of plant height in noninoculated plants.
** Percentage of TYLCV DNA of the STY–1 line.
(Source: Lepidot et al. 2006)

systemic in plants showing resistance to symptoms in a manner that is indistinguishable from the susceptible plants, but disease symptoms are not visible. Such plants are considered as tolerant and this trait is heritable. Tolerance to *Cucumber mosaic virus* has been exploited as a management strategy in cucumber production (Hull 2002). Acquired resistance is another phenomenon unique to virus infection. The susceptible plants, when inoculated with a compatible virus, becomes resistant to subsequent infection by the same virus or its strains. Acquired resistance also known as cross-protection against related strains or viruses has been the principal management strategy adopted in the case of certain viruses infecting fruit trees such as the *Citrus tristeza virus*. Furthermore, as most of the plant viruses depend on biological vectors for their dissemination, resistance to vectors has significant epidemiological significance in determining the incidence and subsequent spread of virus diseases (Narayanasamy 2006).

A virus-coded function and the product of host resistance gene may be involved in the recognition leading to susceptibility or resistance. If the product of resistance gene is directly involved in the recognition event, it may be an inhibitor of virus infection and the resistance may be constitutive in nature. Alternatively, the resistance gene product produced on interaction with the virus factor, may signal the induction of a host defense mechanism leading to the production of compounds inhibitory to virus replication and movement. Plants exhibit different grades of resistance to virus which may operate at different steps of virus replication and movement. Plants show different grades of resistance to viruses and they may operate at different steps of virus replication, such as uncoating, synthesis of replicase, translation of virus-coded information, and production of movement protein.

The presence of over 200 virus *R* genes in crops, their wild relatives and the model plant *Arabidopsis thaliana* has been reported. More than 80% of viral resistance genes identified are monogenically controlled, the rest being under oligogenic or polygenic control. Although more than half of the reported monogenic resistance traits exhibit dominant inheritance, relatively high proportion of recessive viral *R* genes determine the resistance trait in contrast to fungal or bacterial resistance that

is reported to be dominant (Kang et al. 2005b). The *R* genes have been tagged with molecular genetic markers such as RFLP, AFLP, RAPD and other PCR-based markers. As in the case of fungal and bacterial diseases, molecular markers linked to *R* genes may be employed for indirect selection via genotype, for locating *R* genes in plant genomes and for gene isolation. The QTLs that have been tagged for plant viral resistance are less compared to fungal or bacterial disease resistance (Chague et al. 1997; Ben-Chaim et al. 2001; Loannidou et al. 2003).

The response of many *Nicotiana* varieties to *Tobacco mosaic virus* (TMV) by producing necrotic local lesions depends on the presence of a single dominant *N* gene found naturally in *Nicotiana glutinosa* which is regularly used as an assay host to estimate the virus concentration. The virus contents are proportional to the number of local lesions formed on the leaves of *N. glutinosa* inoculated with different concentrations of TMV (Narayanasamy and Doraiswamy 2003). The *N* gene has been incorporated into tobacco cultivars Samsun NN, Xanthi nc, and Burley NN. Programmed cell death (PCD) occurs at the site of infection where virus particles may be detected but restricted to the region immediately surrounding the necrotic lesion (da Graca and Martin 1976). It has been hypothesized that TMV protein interacts either directly or indirectly with N protein, resulting in the activation of the signal transduction pathway leading to the HR. Characterization of *N* gene showed that this gene encoded a protein of 131.4 kDa with a N-terminal domain similar to that of the cytoplasmic domain of *Drosophila* Toll protein and the interleukin-1 receptor in mammals, a nucleotide-binding site (NBS) and four imperfect leucine-rich repeat (LRR) regions. The *N* gene belongs to the TIR-NBS-LRR class of *R* genes (Dinesh-Kumar et al. 2000). The *N* gene has been transgressed into tomato for conferring resistance to TMV (Whitham et al. 1996). Another gene *N′* from *N. sylvestris* has also been demonstrated to control HR against most tobamoviruses except U1 (vulgare) and OM strains that infect plants with N′ gene systemically. Induction of HR by *N′* gene was shown to be due to TMV coat protein (CP) gene (Saito et al. 1987). The mutations that altered the tertiary structure of the CP abolished HR (Culver et al. 1994). The investigation to identify elicitor site in CP of TMV revealed that substitutions that disrupted the right face of the CP, α-helical bundle interfered with N′ gene recognition. It was suggested that the three-dimensional fold of the CP in addition to specific surface features within the elicitor active site appear to determine the N′ gene specificity (Taraporewala and Culver 1997).

Polyamine (PA) metabolism in plants is altered due to interaction with biotic and abiotic stresses. Yamakawa et al. (1998) identified spermines (Spm) among polyamines (PAs), as one of the endogenous inducers for the production of PR-proteins during HR of tobacco carrying N-resistant gene challenged with TMV. In addition, it was shown that exogenous application of Spm resulted in induced expression of PR proteins and enhancement of resistance to TMV. A later investigation showed that Spm led to mitochondrial dysfunction in tobacco followed by the activation of salicylic acid (SA)-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK). The possible downstream components of the Spm signaling pathway was investigated by isolating Spm-responsible genes by differential hybridization approach. The harpin (produced by bacterial TTSS)-induced 1 (*HIN1*)

gene was responsive to Spm. Two novel *HIN1*-like tobacco cDNAs designated *HIN9* and *HIN18* were also isolated by genomic Southern analysis. The gene *HIN1* was found to be upregulated during HR generated by an incompatible pathogen interaction and during senescence. All three *HIN1* family genes were responsive to HR due to TMV infection. As *HIN1* and its closely related genes are Spm-responsive, it is suggested that Spm may have a role as a signal transmitter in the HR formation (Takahashi et al. 2004).

The genes conferring HR to TMV in other crops such as *Capsicum annuum* and tomato have been identified. The *L*1gene in *C. annuum*, L2 in *C. frutescens* and L3 in *C. chinense* confer HR resistance to TMV (Berzel-Herranz et al. 1995; de la Cruz et al. 1997; Dardick et al. 1999). An uncharacterized HR gene in eggplant (brinjal) conferring resistance to TMV was reported by Dardick and Culver (1997). HR to Tomato mosaic virus (ToMV) strains is governed by two allelic genes, Tm-2 and Tm-22 in tomato. The movement protein (MP), with MW of 30 kDa, but not the CP of TMV isolates was shown to be the inducer of HR in both Tm-2 and Tm-22 plants (Weber et al. 1993). However, the later study by Weber and Pfitzner (1998) suggested that both the resistance genes Tm2 and Tm22 appeared to operate via an HR, rather than inhibition of movement of the virus out of the initially infected cell due to incompatibility of the MP. A large number of potyviruses seems to be controlled by the I gene in P. vulgaris in a dominant manner. These viruses are *Azuki mosaic virus, Bean common mosaic virus, Bean necrotic mosaic virus, Blackeye cowpea mosaic virus, Cowpea aphid-borne mosaic virus, Passion fruit woodiness virus K, Soybean mosaic virus, Thailand Passiflora virus, Watermelon mosaic virus* and *Zucchini yellow mosaic virus* (Fisher and Kyle 1994). In addition, the locus conferring resistance to these viruses, was also implicated in modulating a necrotic response to *Bean severe mosaic virus* belonging to Comoviridae.

It has been established that I locus occurs in a large cluster of TIR-NBS-LRR sequences (Kang et al. 2005b). In contrast, two or more loci may be required for the virus resistance response as in bc-u system in bean for resistance to several pathotypes of Bean common mosaic virus (BCMV) (Drijfhout 1978). The R genes involved in resistance to different pathotypes of the same viral species, closely related viral species or diverse plant pathogen groups have been found to be clustered together. Two distinct types of gene clusters have been recognized. The set of genes exhibiting similar pattern of inheritance and resistance phenotypes that control very closely related viral genotypes, form one type of R gene cluster. The second type of R gene cluster encloses viral resistance with R genes that can control unrelated pathogens. The wheat Bdv1 allele that confers resistance to Barley yellow dwarf virus (BYD) was shown to be linked to R genes Lr34 and Yr18 conferring adult plant resistance to rusts in bread wheat (Singh 1993). Likewise, QTL for plant viruses including Potato virus X (PVX) and R gene providing resistance to the nematode (Globodera pallida) were reported to be tightly linked (Van der Vossen et al. 2000).

Resistance conferred by dominant *R* gene is frequently, but not always is associated with HR (Fraser 1990). Possibly this may be due to the use of HR as a diagnostic indicator of field resistance by researchers. Specific recognition of the

virus by the host leads to HR which localizes virus spread by rapid PCD surrounding the infection site culminating in the production of visible necrotic local lesions. The extent of visible HR may be influenced by various factors such as gene dosage, genetic background, environmental conditions such as temperature and viral genotype (Collmer et al. 2000). Among the plant cloned genes (>40) showing monogenic dominant resistance, *N* gene for TMV resistance in tobacco (Whitham et al. 1994), *Rx1* and *Rx2* for PVX resistance in potato (Bendahmane et al. 1995, 2000), and *Sw5* for *Tomato spotted wilt virus* (TSWV) resistance in tomato (Brommonschenkel et al. 2000) have been studied. Many of the recessive genes seem to act at single cell level or affect cell-to-cell movement. Resistance to potyviruses have been reported to be due to recessive *R* genes (Shukla et al. 1994). The melon (*Cucumis melo*) accession TGR-1551 was found to be consistently resistant to *Water melon mosaic virus* (WMV) when inoculated mechanically or via aphid transmission. This accession exhibited resistance to WMV isolates collected from different regions of Spain. The genetic analysis showed that the resistance to WMV in TGR-1551 was goverened by recessive gene. The resistance mechanism did not function at early stages of infection, since WMV accumulated efficiently in inoculated leaves. Virus movement-based form of resistance appeared to be operative in TGR-1551 (Diaz-Pendón et al. 2005).

Two principal types of resistance – hypersensitive and extreme – to *Potato virus Y* (PVY), in potato, have been recognized. The HR to PVY is strain-specific, causing a range of necrotic reactions in both locally and systemically infected leaves (Valkonen et al. 1998). The *Ny* genes causing HR have been identified in several potato cultivars (de Galarreta et al. 1998). But in some varieties, HR may not restrict virus spread in plants (Vidal et al. 2002). The potato chromosome contains the HR gene Ny_{tbr} (Celebi-Toprak et al. 2002). The *Ry* genes for extreme resistance (ER) to PVY, confer extremely high level of protection against all PVY strains (Vidal et al. 2002). The Ry_{adg} (*Solanum tuberosum* ssp. *andigena*) and Ry_{sto} (*S. stoloniferum*) were mapped to chromosome XI whereas RY_{chc} (*S. chaoense*) was located in chromosome IX (Hämäläinen et al. 1997; Brigneti et al. 1997; Hosaka et al. 2001). Marker-assisted selection (MAS) has been found to be one of the most useful applications of DNA-based markers in potato breeding programs for disease resistance. A novel locus for ER to PVY, Ry-f_{sto} was identified on potato chromosome XII. Bulked segregant analysis (BSA) was applied to identify an inter-simple sequence repeat (ISSR) marker $UC857_{980}$ linked to Ry-f_{sto}. This marker mapped to linkage group XII of a reference potato RFLP map. The RFLP markers were converted into PCR-based sequence tagged site (STS) and cleaved amplified polymorphic sequence (CAPS) markers. The CAPS marker $GP122_{718}$ was tightly linked to the resistance gene that was reliably identified in Polish and German cultivars expressing ER to PVY. The results indicated that the CAPS marker $GP122_{718}$ has the potential for use as a diagnostic tool to monitor the distribution of specific resistance gene in a wide gene pool (Flis et al. 2005).

Bean common mosaic virus (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are seed-transmitted at high frequency, making most disease management strategies ineffective. The use of resistant cultivars appears to be the only

feasible approach for effective management of these two viruses. Use of molecular markers has been demonstrated to accelerate progress in breeding programs. The *bc-1*^{2} allele conferred resistance to BCMV stains within pathogroups I, II and V and pathogen group III strains of BCMNV (Drijfhout 1978). The usefulness of sequence characterized amplified region (SCAR) markers which are tightly linked with *bc-1*^{2} were developed (Miklas et al. 2000; Vandemark and Miklas 2002). The SCAR marker linked to *I* allele which conditioned resistance to all strains of BCMV and BCMNV was developed by Melotto et al. (1996). For simultaneous genotyping of bean plants for the *I* and *bc-1*^{2} alleles which condition resistance in bean to BCMV and BCMNV, a multiplex real-time PCR assay was shown to be effective. The segregating bean populations could be used for assigning genotypes for both the *bc-1*^{2} and *I* alleles. The number of F3 family progeny tests was substantially reduced. Furthermore, the desired genotypes can be identified at the seedling stage resulting in acceleration of progress in germplasm enhancement and cultivar development (Vandemark and Miklas 2005).

Screening of 122 early maturing European maize inbred lines for resistance to *Sugarcane mosaic virus* (SCMV) and *Maize dwarf mosaic virus* (MDMV) indicated that three inbreds (D21, D32 and FAP 1360A) were completely resistant under field and greenhouse conditions (Kuntze et al. 1997). Two major QTL regions, *Scmv1* and *Scmv2* conferring resistance to SCMV were mapped to chromosome arms 6S and 3L (Dussle et al. 2000; Xu et al. 2000). The genes that were consistently expressed differentially between resistant and susceptible inbreds were identified, using a macroarray-based expression patterns in maize inbred lines. The change of RNA profiles was monitored on a macroarray containing suppression subtractive hybridization (SSH) clones. The number of differentially expressed genes (SCMV infected vs. non-infected) in individual lines was variable in the seven inbreds tested. The candidate genes (112) were grouped into three clusters. Five genes surrounding *SCMV2* QTL provided a better understanding of signal transduction pathway of or SCMV resistance. They have the potential to be employed as functional markers for distinguisting resistant and susceptible maize genotyping (Shi et al. 2005).

Genetic resistance to certain viral families like *Geminiviridae* may be difficult to locate and it may be highly strain-specific and/or quantitatively inherited. Hence, considerable difficulty may be posed in developing resistant cultivars and to develop molecular markers. Members of the genus *Begomovirus* have monopartite (one DNA) or bipartite (2 DNAs – DNA-A and DNA-B) genomes and are transmitted by whiteflies. They infect dicotyledonous plants such as common bean, pepper, tomato, melon and cotton. *Bean dwarf mosaic virus (BDMV)* infecting bean (*Phaseolus vulgaris*) is well characterized at both biological and molecular levels. The bean cultivar such as Black Turtle Soup (BTS), Olathe, Othello, and Pinto UI 114 were found to be resistant to BDMV. By employing a BDMV-GFP reporter, blocking of long-distance movement of the virus was possible in cv. Othello. In addition, resistance was associated with a HR in vascular tissues which provided an easily scorable marker of resistance phenotype (Wang et al. 1999a; Garrido-Ramirez et al. 2000b). But BDMV resistance in cv.BTS also depended on a block

in long-distance movement, and not in the HR as in cv. Othello (Garrido-Ramirez et al. 2000a). In a later study, the Middle American germplasm representing four recognized races and the parents of Othello were inoculated with BDMV and a BDMV-green fluorescent protein (GFP) reporter. Partial or complete resistance was evident in the entries tested, indicating the availability of several screens of BDMV resistance in the Middle American gene pool. Many BDMV resistant germplasm types did not react with HR, indicating that HR was not correlated with resistance to this virus. BDMV resistance involved a block in viral long distance movement and did not require HR. This resistance was conferred by a single dominant allele. The HR may be a secondary defense response that develops subsequent to the initial defense response (Seo et al. 2004).

The bean leaf crumple disease (BLCD) is due to the *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Málaga virus* belonging to *Begomovirus*. Two bean breeding lines GG9 and GG12 showed resistance to BLCD-associated viruses. The GG12 plants exhibited an equivalent resistance response under natural (*Bemisia tabaci*-mediated) or experimental (*Agrobacterium tumefaciens*–mediated) inoculations. Genetic analysis suggested the resistance to BLCD to be due to a single dominant gene. This resistance provided complete protection as no symptoms could be seen under field conditions or after experimental inoculation. Virus replication in GG12 was not inhibited in the inoculated leaves, but a severe restriction of systemic accumulation BLCD-associated viruses was observed. The cell-to-cell or long-distance movement of the viruses may occur. In addition, the inoculated plants recovered from virus infection, indicating the possible progressive reduction in virus population with lapse of time after inoculation (Monci et al. 2005).

The genetic basis of resistance to *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mosaic virus* (ToMV) was investigated. Partially dominant *Ty-1*and *Tylc* derived from *Lycopersicon pimpinellifolium* and two other genes from *L. chinense* were reported to confer resistance to TYLCV (Pilowsky and Cohen 1984; Zamir et al. 1994; Gomez and Laterrot 1997). Wild tomato accessions of *L. chinense* had high level of resistance to ToMV (Scott and Schuster 1991). Many RFLP and RAPD markers have been shown to be linked to TYLCV and ToMV resistance genes (Zamir et al. 1994; Griffiths and Scott 2001). In a later investigation, bulked segregant analysis (BSA) was applied to identify RAPD markers linked to TYLCV and ToMV resistance. Multiple-QTL mapping (MQM) was employed to identify QTL for resistance linked to chromosome 6. Saturation mapping of RAPD markers on chromosome 6 surrounding the resistance genes in *L. chinense* was useful to identify markers more tightly linked to resistance. Three different sources of TYLC and ToMV resistance and the markers tightly linked to them were identified, providing a means of systematically combining multiple resistance genes. This study has brought to light the diagnostic tools that could be developed to screen tomato populations for the presence of *R* genes rapidly and efficiently in a cost-effective manner (Agrama and Scott 2006).

Occurrence of big vein disease of lettuce was known for many years. However, the casual virus *Mirafiori lettuce big vein virus* (MLBVV, *Ophiovirus*) formerly

known as *Mirafiori lettuce virus,* was identified much later (Lot et al. 2002). Another virus *Lettuce big-vein-associated virus* (LBVaV, *Varicosavirus*) formerly designated *Lettuce big-vein virus* was shown to be associated with big vein disease. Frequently plants exhibiting big vein symptoms were found to be coinfected with both viruses. Among the wild relatives of lettuce accessions of *Lactuca virosa*, when inoculated, did not develop any symptom of infection. *L. virosa* accession IVT280 was shown to be asymptomatic entirely (100%) in the greenhouse inoculation trials. RT-PCR and nucleotide sequencing were performed to determine infection with MLBVV and LBVaV and sequence diversity among viral isolates respectively. Analysis of RT-PCR showed no viral amplification in the accession IVT 280, indicating its apparent immunity to the viruses. The lettuce cultivars Margarita and Pavane which were considered resistant earlier, accumulated both MLBVV and LBVaV (Table 3.4). The RT-PCR assay developed in this study demonstrated MLBVV accumulation in symptomless plants (termed resistant based on symptom expression) can greatly help breeding efforts for big vein disease resistance derived from *L. virosa* accession (Hayes et al. 2006).

Cotton leaf curl disease (CLCuD) is caused by a begomovirus in association with ss-DNA satellite designated DNAβ (Mansoor et al. 2003) which is required to infect and produce symptoms in cotton. The satellite is considered to suppress host defense responses, but requires the helper virus for its trans-replication, movement in plants and in seed transmission (Saunders et al. 2004). Crosses between the highly susceptible cotton variety (S-12) and highly resistant varieties (CP-1512, LRA-5166 and (IM-443) were effected. Viral infection was determined by visual observation, dot-blot and multiplex PCR assays. Of the 467 symptomless F2 plants (CP-15/2 × S-12) the virus was detected in 364 plants by multiplex PCR. The symptomless virus-infected plants were placed under 'resistant' category and the PCR-negative plants under 'highly resistant' category. The F3 progeny of susceptible F2 plants segregated for resistance, indicating the probable operation of suppressor gene (S). It was suggested that three genes may be involved in the resistance of *G. hirsutum* to CLCuD, two for resistance and one as a suppressor of resistance (Rahman et al. 2005).

Resistance to the natural vectors of plant viruses in addition to resistance to the virus themselves will be the ideal strategy to combat crop virus diseases effectively. Wilson et al. (1989) considered the resistance to *Cotton leaf crumple virus* (CLCrV) in the cotton cultivar Cedix could be exploited. Cotton genotypes and cultivars were evaluated for their resistance to CLCrV and the whitefly vector *Bemisia tabaci*. None of the entries showed high level of resistance to *B. tabaci.* The entries AP6101 and AP4103 had the lowest whitefly densities, but they showed highest CLCr disease ratings too, indicating that there may not be any relationship between whitefly density and resistance to CLCr disease. The genotypes NK2387C and DPX 1883 were considered as highly resistant to CLCrV, since no symptom could be seen. In addition, the virus could not be detected by highly sensitive PCR assay. The resistant entries were of cv. Cedix parentage. The dot-blot technique employing cloned CLCrV DNA as a probe estimated viral titer efficiently in cotton germplasm. The viral titer was negatively correlated with disease resistance (Seo et al. 2006).

Table 3.4 Detection of MLBVV and LBVaV in symptomatic and asymptomatic lettuce plants using RT-PCR assay

Test genotypes/lines	Symptomatic plants				Asymptomatic plants				
	RT-PCR response				RT-PCR response				
	No. of Plants	MLBVV positive	LBVaV positive	MLBVV +LBVaV positive	No. of Plants	Virus negative	MLBVV positive	LBVaV positive	MLBVV +LBVaV positive
Great Lakes 65 uninoculated	0	0	0	0	7	7	0	0	0
Great Lakes 65 inoculated	5	0	0	5	2	0	0	1	1
Magarita inoculated	6	3	0	3	7	3	0	3	1
L. virosa IVT 280 – inoculated	0	0	0	0	14	14	0	0	0

(Source: Hayes et al. 2006)

3.3.2 Molecular Basis of Resistance to Viral Diseases

The life cycle of plant viruses is very short compared to cellular plant pathogens. Yet they undergo a multistep process involving entry into plant cells, uncoating of nucleic acid (NA), translation of viral proteins, replication of viral NA, assembly of progeny virions, cell-to-cell movement, systemic movement and plant-to-plant transmission. The incompatible interaction between host plant and virus may be predominantly due to dominant resistance and in some cases due to recessive resistance. In several pathosystems, dominant resistance results from inactive recognition event that may occur between host and viral factors, leading to induction of host defense responses. The molecular biology of this recognition is not clearly understood.

3.3.2.1 Resistance to Virus at Cellular Level to Viral Disease

Cellular level resistance may be characterized as a state where virus replication is not likely to occur or occurs at an undetectable level in inoculated cells. This type of resistance is observed in cowpea cv. Arlington challenged with *Cowpea mosaic virus* (CPMV). The involvement of a protease inhibitor in preventing CPMV polyprotein processing was suggested for the inhibition of virus replication (Ponz et al. 1988). The host translation factors have been implicated in the resistance of pepper to *Tobacco etch virus* (Kang et al. 2005a) and barley to *Barley yellow mosaic virus* complex (Pellio et al. 2005). Another type of mechanism occurring at cellular level involves an active resistance response to virus infection that limits virus replication rapidly before cell-to-cell movement is initiated. No symptoms or extremely limited necrosis may be seen in such plants. Resistance offered by *Tm-1* against TMV in tomato (Watanabe et al. 1987), by *Nx* and *Rx* against PVX and *Ry* against PVY in potato (Solomon-Blackburn and Barker, 2001), by *Sw5* against TSWV in tomato (Brommonschenkel et al. 2000) and by *Rsv1* against *Soybean mosaic virus* in soybean (Hajimorad and Hill 2001) has been reported. The host response of this type of extreme resistance (ER) in potato revealed the absence of any visible symptoms when challenged with PVY. By applying RNA hybridization and ELISA, accumulation of PVY could not be detected in inoculated leaves of potato carrying *Ry* gene. In addition, the protoplasts isolated from these plants did not support replication of PVY. As no HR could be seen, it was hypothesized that inhibitors of virus accumulation might be encoded by the resistance genes.

3.3.2.2 Resistance to Virus Movement Within and Between Cells

In some pathosystems, the virus may be able to establish infection in or a few cells, but the virus fails to move to other cells because of failure of interactions between plant and viral factors required for cell-to-cell movement or due to active host defense responses that may rapidly restrict virus spread. In the case of DNA viruses, crossing the nuclear membrane is a formidable barrier for virus movement (Whittaker and Helenius 1998). The viral proteins needed for import of virus into the nucleus, and export of progeny genomes back to the cytoplasm for translation

and virion assembly have been identified for some viruses. The nuclear import and export of bipartite geminivirus DNA was shown to be mediated by the BV1 (BR1) protein (Carrington et al. 1996). But the host factors that can interfere with viral protein functions resulting in resistance have not yet been identified. Hypersensitive response (HR) is one of the mechanisms that serves to disrupt cell-to-cell movement of plant viruses. However, HR is not correlated with resistance as in the case of *Bean dwarf mosaic virus* (BDMV) (Seo et al. 2004).

In *Nicotiana* plants carrying N-gene resistance to TMV, hypersensitive cell death occurred to form synchronous necrotic lesions. Four vacuolar processing enzymes (VPE) cDNAs were isolated from TMV-infected leaves. Both mRNA and protein levels of VPE were rapidly and transiently increased at an early stage of HR in leaves. Necrotic lesion formation was strongly inhibited by a VPE inhibitor Ac-ESSE-CHO and a caspase inhibitor Ac-YVAD-CHO. The results suggest that VPE activity and caspase-1 activity contributed to TMV-induced cell death. VPE is structurally unrelated to caspases whose activity is required for programmed cell death (PCD) in animals. However, VPE has been shown to have a caspase-1 activity. Thus plants appear to have a regulated cellular suicide strategy, that unlike PCD of animals, is mediated by VPE and the cellular vacuole (Hatsugai et al. 2004).

All tobamoviruses that elicit the L^2-gene-mediated HR in pepper plants, also elicit HR in L^3 plants. But *Pepper mild mottle virus* (PMMO) elicits the resistance conferred by the L^3 gene, but overcomes L^2-gene-mediated resistance. In order to identify viral factors affecting induction of HR-mediated by the *Capsicum* spp., chimerical viral genomes between *Paprika mild mottle virus* (PaMMV) (a virus capable of inducing HR) and PMMoV were constructed. A hybrid virus with PaMMV-CP gene substituted in the PMMoV-S sequence was able to elicit HR in *C. frutescens* (L^2L^2) plants. The results indicated that the sequences affecting induction of the HR-mediated by the L^2-resistance gene resided in the CP gene. The elicitation of the host response depends on CP, but not on the RNA (de la Cruz et al. 1997).

Majority of *R* gens has been shown to encode a nucleotide-binding (NB) site/leucine-rich repeat (LRR) protein and they confer resistance to diseases caused by fungal, bacterial and viral diseases of plants. The LRR domains mediate recognition of pathogen-derived elicitors that induce defense-related responses of host plants. A random in vitro mutation analysis was performed to understand how mutations in an R protein (*Rx*) LRR domain generate disease resistance specificity. Potato plants with *Rx* gene showed resistance to strains of *Potato virus X* (PVX) with threonine and lysine at positions 121 and 127 of the elicitor CP (CP-TK), but not those with lysine and arginine (CP-KR). Selected mutants were able to protect potato plants against an additional strain of PVX and also against the distantly related *Poplar mosaic virus*. The investigation has opened up the possibility of exploiting *R* genes through effective manipulation for enhancing resistance to a spectrum of virus (Farnham and Baulcombe 2006). The role of recessive genes in the movement of viruses has been indicated in certain pathosystems. The movement of *Tobacco vein mottling virus* and PVY was found to be controlled by the recessive gene *va* (Gibbs et al. 1989).

The cell-to-cell movement of plant virus may be hampered by the HR of the infected plant. A cascade of host defense responses is induced following recognition of the viral elicitor. These responses include oxidative H_2O_2 bursts and up-regulation of hydrolytic enzymes, PR proteins and callose biosynthesis. The activation of tobacco *N* gene and tomato *Tm-2* and *Tm-2*^{2} alleles results in restriction of viral movement within a small zone of small number cells (Otsuki et al. 1972; Motoyoshi and Oshima 1975). No cell death was evident in protoplasts isolated from tobacco plants carrying *R* genes, although TMV multiplication was not affected. Necrotic cell death is considered as an ancillary consequence of resistant response not required for the suppression of the viral replication. Similar results were obtained in *Phaseolus vulgaris* carrying the allele *I* challenged with *Bean common mosaic virus* (Collmer et al. 2000). The absence of resistance being consequence of HR was also reported in the case of the *defense no death* (*dnd1*) mutant of *Arabidopsis* (Yu et al. 1998).

Resistance to *Clover yellow vein virus* (ClYVV) in two pea lines PI 347295 and PI 378159 was shown to be due to a single recessive gene. In order to monitor spread of infection by ClYVV, one of the isolates (No. 30) was tagged with a green fluorescent protein (GFP). Although the virus replicated at single cell level in both pea lines, the nature of virus movement showed variation. The virus did not move to adjacent cells in PI 347595, indicating resistance operated at cell-to-cell movement step. On the other hand, in PI 378159, ClYVV was able to move to adjacent cells around infection site and reach the leaf veins. But the virus moved at a slower pace compared with susceptible pea line. The GFP tagging technique was found to be an efficient tool for characterizing the two resistance modes to ClYVV in pea lines (Andrade et al. 2007).

3.3.2.3 Resistance to Long-Distance Movement

Systemic infection represents the susceptibility response of the host plant. Plasmodesmata are elaborate and highly regulated structures with which viruses can interact for both cell-to-cell and long distance movement, establish symplastic connectivity between the epidermal/mesophyll cells and cells with the vascular elements, including sieve elements (SE) (Lucas and Gilberton 1994; Carrington et al. 1996; Santa Cruz 1999). The virus entry into the SE-companion cell appears to be a barrier. After entry into companion cell, the virus may have direct access to the sieve tube, the conducting element of the phloem through which nutrients and viruses have to move. Only a few host factors that may affect the long distance movement of viruses have been identified. Long distance of movement of TMV was impaired due to down-regulation of pectin methyl esterase (PME) resulting in blocking of virion exit from phloem (Chen and Citovsky 2003). Mechanisms negatively affecting systemic movement of viruses have been recognized. Long distance movement of *Tobacco etch virus* (TEV) in V2O tobacco was significantly reduced because of reduced entry into and exit from SE. The interaction of two unlinked, unidentified recessive genes control the movement of TEV in SE (Schaad et al. 1997). Entry of *Cowpea chlorotic mottle virus* (CCMV) into vascular tissue was restricted in

soybeans homozygous for two recessive genes (Goodrick et al. 1991). Likewise, vascular movement of *Potato virus A* was entirely blocked in potato with homozygous recessive *ra* allele (Hämäläinen et al. 2000). The rate of systemic movement of virus may markedly reduce in some pathosystems as an expression of resistance. Resistance to *Potato leafroll virus* (PLRV) was associated with an exclusion of virus from external phloem bundles (Derrick and Barker 1997), whereas disrupted movement of *Cucumber mosaic virus* in phloem tissue and confinement of CMV in the lower portions of stems of resistant pepper plants were considered to contribute to the resistance against the virus (Dufour et al. 1989). The genes involved in protection of plants against these viruses remain to be identified.

Avirulence genes have been recognized by their requirement for disease resistance in plants possessing corresponding *R* genes. Large numbers of genomes of a single virus may be present in a single host plant cell across many cells per host. Avirulence determinants are conventionally identified by producing chimeric clones derived from viral genotypes with different virulence levels followed by assessment of their infectivity on suitable host plant species. Avirulence factors with respect to *R* genes have been shown to be virtually any part of the viral genome conferring resistance against the virus concerned. The viral RNA polymerase subunits, movement protein and CP of different viruses may function as avirulence determinants. The HC-Pro and P3 proteins of *Soybean mosaic virus*, function as avirulence determinants for *Rsv1* in soybean (Eggenburger and Hill 1997). In the case of *Turnip mosaic virus,* the cylindrical inclusion (CI) and P3 proteins function as avirulence determinants for the *Brassica napus* R genes, *TuRBO1* and *TuRBO4/5* (Jenner et al. 2000, 2002, 2003).

The evaluation of virus resistance using molecular markers has been successful in several crops. Biochemical markers associated with disease resistance would be very useful for accelerating selection of resistant plants or sources of resistance to viruses affecting perennial horticultural crops. *Plum pox virus* (PPV) causes the Sharka disease affecting *Prunus* spp. including apricot and peach seriously. Apricot cultivars showing resistance to PPV had higher activities of catalase, ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) compared to susceptible cultivars, suggesting the usefulness of the activities of these enzymes as biochemical markers for Sharka (Hernández et al. 2006). The possibility of using the differential response of the apoplastic antioxidant system in resistant and susceptible cultivars of apricot as biochemical markers, following inoculation with PPV was explored. In the resistant apricot cultivar (Stark Early Orange), an increase in Class I ascorbate peroxidase (APX) and a strong increase in peroxidase (POX) and superoxide dismutase (SOD) was observed in the apoplastic compartment. Increase in appoplastic H_2O_2 levels in leaves of susceptible plants led to increase in electrolyte leakage which was absent in resistant plants (Diaz-Vivancos et al. 2006).

The defense responses of potato cv. Desireé to infection by *Potato virus X* (*PVX*) at the transcript and metabolite level were investigated. Salicylic acid (SA) biosynthesis and expression of several defense genes including PR-1 and glutathione – S–transferase (GST), involved in ET-and ROS-dependent signaling were highly up-regulated in upper uninoculated (systemic) leaves of PVX-infected potato plants

compared with mock-inoculated control plants. In addition, the β-phenylethylamine alkaloids such as tyramine, octopamine and norepinephrine were found to be highly induced upon PVX infection. These alkaloids may possibly, be involved in the elicitation of plant defense responses by forming hydroxycinnamic acid amides (HCAA) which are considered to enhance cell wall stability by extracellular peroxidative polymerization. Infection by PVX markedly increased the expression of tyramine-hydroxycinnamoyl transferase (THT) and apoplastic peroxidase (POD) in systemic leaves. The metabolic and transcriptional changes observed in PVX-infected potato plants might represent the SAR response against subsequent pathogen infection (Niehl et al. 2006).

3.3.2.4 Posttranscriptional Gene Silencing (PTGS)

An important mechanism known as RNA silencing is involved in gene regulation in several organisms including plants. A major break through in the history of gene silencing happened when a gene silencing response was discovered in the nematode *Caenorhabiditis elegans*. The experimentally introduced ds RNA led to the loss of expression of the corresponding cellular gene by sequence-specific RNA degradation mechanism (Fire et al. 1998). Posttranscriptional gene silencing is an RNA silencing-based approach employed to reduce the level of expression of a (viral) gene of interest. RNA silencing may be subcalssified into RNA-mediated transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). RNA-mediated TGS occurs when ds-RNA with sequence homology to a promoter is produced, leading to de novo DNA methylation of promoter region of the structural gene (Mette et al. 2000). On the other hand, PTGS leads to reduced steady state levels of targeted host or viral cytoplasmic RNA and to a lesser, but sometimes detectable reduction in nuclear RNA. PTGS may be mediated through a host-encoded RNA-dependent RNA polymerase (RdRP) and RNA helicase (Dalmay et al. 2000, 2001). RNA silencing is conserved in a wide range of eukaryotes and is manifested as 'quelling' in fungi (*Neurospora crassa*), RNA interference (RNAi) in animals and cosuppression or posttranscriptional gene silencing (PTGS) in plants. The unifying characteristic of RNA silencing is the presence of 21 to 26-nucleotide (nt) small interfering RNAs (Hamilton and Baulcombe 1999; Hamilton et al. 2002).

The PTGS in plants inactivates some aberrant or highly expressed RNAs in a sequence-specific manner in the host cell cytoplasm and it is an innate antiviral defense in plants (Soosaar et al. 2005). Many RNA and DNA viruses stimulate PTGS shortly after infection, because of the formation of double-stranded structures (transient replication intermediates). Plants transformed with constructs that produce RNAs capable of duplex formation induce virus immunity with almost 100% efficiency, when targeted against viruses (Smith et al. 2000; Wang et al. 2000; Kalantidis et al. 2002). Regardless of the origin, the occurrence of ds RNAs in the cytoplasm of plant cells induces PTGS. RNA-dependent RNA polymerase has also been shown to be involved in PTGS (Dalmay et al. 2000). Genes silenced by PTGS continue to be transcribed. However, their polyadenylated transcripts become nearly undetectable due to the action of a sequence specific RNA degradation mechanism.

PTGS can generate mobile silencing signals with sequence-specific information that spreads from cell-to-cell through plasmodesmata and systemically through vascular system to different plant organs (Mlotshwa et al. 2002; Himber et al. 2003).

Plant viruses are known as strong inducers as well as targets of PTGS. Following virus infection, virus-specific ds RNA forms were isolated from infected plant tissues (Díaz-Ruíz and Kaper, 1978). As ds-RNA does not naturally occur in the cytoplasm of plant cells, recognition of nucleic acid structures that are present only in infected cells appears to induce a natural line of defense against viral infections (Voinnet 2001). Virus-induced gene silencing (VIGS), a characteristic manifestation of PTGS in which viruses are both triggers and targets of silencing, has evolved into a viral surveillance system triggered by ds-RNA produced during the intermediate steps of virus replication (Waterhouse et al. 2001). Accumulation of 21-nt ds siRNAs occurs during viral infection in inoculated leaves and other tissues far away, indicating the activation of PTGS. Enhanced levels of siRNA have been correlated with a reduction in titer of the virus and in some pathosystems, immunity or recovery in upper noninoculated leaves results (Szittya et al. 2002). PTGS has the potential to be an RNA-mediated defense response to protect plants against plant viruses (Moissiard and Voinnet 2004). However, the viruses in compatible interaction, are able to counteract RNA silencing, by producing suppressor proteins that suppress various steps of the silencing machinery (Silhavy and Burgyan 2004). Introduction of transgenes constitutively expressing part of the genome of virus may lead to resistance of the plant to infection by the virus concerned (Marathe et al. 2000). Plants may become resistant if the transgene undergoes PTGS prior to infection. On the other hand, plants in which the trangene undergoes PTGS after infection, exhibit recovery. Both resistant plants and plants that recover after inoculation, are resistant to secondary infection by the same virus or another recombinant virus carrying part of the genome of the first virus indicating that plants appear to have a 'memory' of the first virus (Vance and Vaucheret 2001).

Intensive research efforts have resulted in the better understanding of the molecular mechanism of PTGS. Both PTGS and RNAi are manifestations of a broader set of gene silencing phenomena common to most eukaryotes. In the PTGS-associated antiviral response, viral ds-RNA is first processed by an RNase III–like nuclease (designated DICER in *Drosophila melanogaster* and for which several homologs in *Arabidopsis* have been identified) into 21-25nt ds-RNAs [small interfering RNAs (si RNAs)] that guide another nuclease complex (RNA-induced silencing complex, RISC) to cleave the RNA genome of the invading virus (Hamilton and Baulcombe 1999; Tang et al. 2003). DICERs can process ds-RNAs into two functionally different small RNAs, micro-RNAs (miRNAs) and small interfering RNAs (siRNAs). The miRNAs are involved in the control of many endogenous protein-encoding mRNAs. On the other hand, siRNAs mainly have a role in suppressing molecular parasites such as transposons, transgenes and viruses (Baulcombe 2004). In the case of *Cymbidium ringspot tombusvirus*, (CymRSV), siRNA sequences have a nonrandom distribution along the length of viral genome, suggesting that these are hot spots of virus-derived siRNA generation. The results suggest that virus–derived siRNAs originate predominantly by direct DICER cleavage of imperfect

duplexes in the most folded regions of the positive strand of the viral RNA (Molnár et al. 2005). The primary principle of RNAi technology is the ability to deliver ds-RNA as potent activator of RNA silencing into an organism or cell, with the aim of triggering sequence-specific degradation of homologous target RNAs. The ds-RNA may be delivered by transforming plants with transgenes that can express a self-complementary RNA. Hybridization occurs with the resulting transcripts itself to form a hairpin structure that consists of a single-stranded loop region (that corresponds usually to an intron) and a base-paired stem, which mimics the ds-RNA structure that induces RNAi (Smith et al. 2000; Kalantidis et al. 2002). The difficulty associated with transformation of some crop plants may be a major limitation in exploiting this RNAi technology for developing crop cultivars resistant to virus diseases.

The potential of ds-RNA-mediated interference in plant virus infections has been assessed to exploit this phenomenon for the control crop virus diseases. Three viruses *Pepper mild mosaic virus* (PMMoV), *Tobacco etch virus (TEV)* and *Alfalfa mosaic virus* (AMV) belonging to tobamovirus, potyvirus and alfamovirus respectively were included in the investigation. Triggering of RNAi could be successfully demonstrated by mechanically inoculating the host plants with in vitro transcribed dsRNA to leaf cells together with the target virus (Tenllado and Díaz-Ruíz 2002). Homologous ds-RNA was able to interfere with virus infection in a systemic host infection. The ability of ds-RNA to specifically interfere with virus infection appeared to require a minimum length of ds-RNA. Tobacco plants inoculated with a mixture containing infectious TEV genomic RNA transcripts and TEV HC-Pro-derived ds-RNA were protected against disease symptoms. In the inoculated plants, no accumulation of viral RNA could be detected either in the inoculated leaves or in upper uninoculated leaves. Similar results were obtained with a mixture of in vitro transcripts of AMV RNAs 1, 2, and 3 plus a ds-RNA covering a 1124-nt region of AMV RNA3 inoculated on *Nicotiana benthamiana* plants none of which developed symptom of infection. Further AMV RNAs were not detected in the test plants (Tenllado et al. 2004).

A simple, fast, safe and inexpensive technique to produce large amounts of ds-RNA derived from viral sequences using an inducible expression system based on a genetically modified *Escherichia coli* strain HT115 (DE3) was developed by Tenllado et al. 2003). The 54-kDa ds-RNA of PMMoV expressed in *E. coli* when sprayed on *N. benthamiana* leaves, was able to interfere with PMMoV infection. The interfering activity of bacterial crude preparations obtained by lysing cell pellets with a French Press was determined. The viral accumulation was entirely inhibited in *N. benthamiana* leaves that were inoculated with a mixture of a bacterial lysate expressing PMMoV-derived ds RNA and PMMoV particles. (Fig. 3.5). Encouraging results were obtained when RNAi was applied against *Plum pox virus* (PPV) causing Sharka or plum pox disease in *Prunus spp.* in an experimental host *N. benthamiana*. A high proportion of plants inoculated with combinations of PPV plus homologous dsRNA-expressing preparations, did not exhibit symptoms of the disease. By applying ELISA and RT-PCR diagnostic tests, no traces of PPV replication could be discerned. This simple method of preparing ds-RNA and easy delivery system

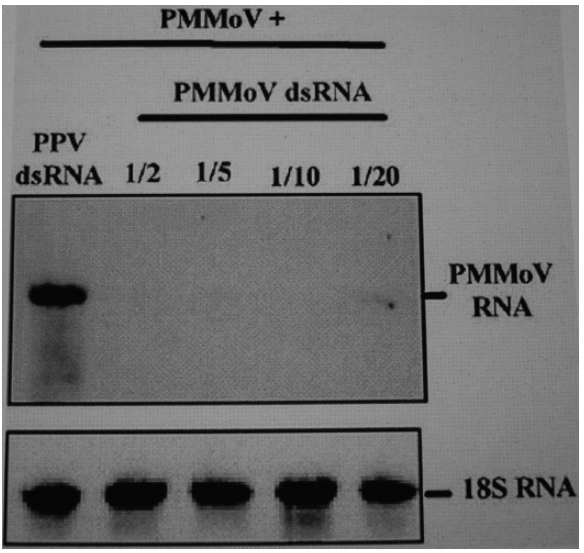

Fig. 3.5 Northern blot analysis of total RNA extracted from leaves of *Nicotiana benthamiana* inoculated with *Pepper mild mottle virus* (PMMoV), mixtures of PoMMV plus PMMoV ds-RNA or *Plum pox virus* ds-RNA (Courtesy of Tenllado et al. 2004; Elsevier, Oxford, UK)

of spraying the RNAi inducers make this technology an attractive alternative strategy for effectively protecting crop plants against several viruses in the near future (Tenllado et al. 2004).

If the silencing signal moves through the plant either with or ahead of an invading virus, it may have an antiviral role of silencing. The silencing machinery in cells receiving the signal could then be primed to target the viral RNA as it enters the cell, so that virus movement through the plant would be impeded. Systemic movement of *Potato virus X* (PVX) into the growing point of infected plants was shown to be dependent on a host-encoded RNA-dependent RNA polymerase (RDR6) which formed an integral part of the RNA silencing machinery (Belkhadir et al. 2005). The RDR6 in *Nicotiana benthamiana* was involved in defense against PVX at the level of systemic spread and in exclusion of the virus from the apical growing point. The RDR6 did not affect primary replication and cell-to-cell movement of the virus. In addition, it had no significant influence on the formation of virus-derived small interfering (si) RNA in a fully established PVX infection. The results suggested that RDR 6 may use the incoming silencing signal to generate double-stranded RNA precursors of secondary siRNA. The secondary siRNAs may mediate RNA silencing as an immediate response that slows down the systemic spread of the virus into the growing point and newly emerging leaves (Schwach et al. 2005).

The movement protein of PVX P25 was suggested to function also as suppressor of silencing through random mutation analysis. The PVX silencing suppressor P25 was shown to be required for cell-to-cell movement of the virus. All mutants that were defective for silencing suppression were nonfunctional in cell-to-cell move-

ment of the virus. In one P25 mutant, proteins that were functional as silencing suppressor, but not as movement proteins were identified. The results indicated that suppression of silencing was not sufficient to allow virus movement resulting in systemic spread of the virus (Bayne et al. 2005). The silencing suppressor protein PO of two *Arabidopsis*-infecting poleroviruses interacted by means of conserved minimal F-box motif with *A. thaliana* orthologs of S-phase kinase-related protein 1 (SKP1), a component of the SCF family of ubiquitin E3 ligases. Point mutations in the F-box-like motif abolished the PO-SKP1 ortholog interaction diminished virus pathogenicity and inhibitied the silencing suppressor activity of PO. Knockdown of expression of a SKP1 ortholog in *Nicotiana benthamiana* rendered the plants resistant to polerovirus infection. The results suggest a model in which PO acts as an F-box protein that targets an essential component of the host PTGS machinery (Pazhouhandeh et al. 2006).

The expression of marker genes of three defense pathways during infection of *Cauliflower mosaic virus* (CaMV) on *Arabidopsis thaliana* a compatible host was analyzed, using luciferase reporter transgenes and directly measuring transcript abundance. The defense response pathways that utilize SA, JA/ethylene and ROS signaling intermediates were activated in this compatible interaction. Expression of PR-1, a marker for SA signaling, was very low until 8 dpi and then rose dramatically, coinciding with virus titers. In contrast, transcriptional up-regulation of glutathione-S-transferase (GST1), a marker for ROS and PDF1.2, a marker for JA/ET defense signaling, could be detected in the virus-inoculated leaf and systemically as early as 2 hpi. In addition, H_2O_2 also accumulated locally and systemically in virus-inoculated, but not in mock-inoculated plants. However, in plants inoculated with infectious CaMV DNA rather than intact virions, the onset of systemic luciferase activity was delayed by 24–48 h, suggesting that virion structural proteins act as the elicitor. This phenomenon of rapid systemic response (RSR) preceded virus movement from the inoculated leaf. Hence, the systemic signal may not be of viral origin. RSR elicited by CaMV may be an aspect of a common response associated with pathogen-associated molecular patterns (PAMPs) – dependent activation of basal response. H_2O_2 accumulation was abolished in NADPH oxidase double mutants (rbohDF) and in *etr1-1* and *ein 2-1* mutants implicating NADPH oxidase and ET signaling in generation and transduction of the response. These results implicate ROS and ET in signaling in response to CaMV infection, but suggest that SA does not play an effective role (Love et al. 2005).

RNA silencing, an ancient mechanism of gene regulation with an antiviral defense role in plants induced by RNA viruses has been better understood compared with DNA viruses. Interactions between DNA viruses and the host plant silencing mechanisms have to be investigated more intensively. *Cauliflower mosaic virus* (CaMV) is a DNA virus expressing its genome through a polycistronic 35S RNA that carries an unusually extensive secondary structure known as translational leader. Analysis of the interaction between CaMV and RNA silencing machinery of *Arabidopsis* and turnip was performed. CaMV-derived siRNAs accumulated in turnip-and *Arabidopsis*-infected plants. The leader was found to be a major, but not exclusive, source for the siRNA molecules. The coordinated and hierachical

action of four *Arabidopsis* Dicer-like (DCL) proteins (DCL1 to DCL4) was required for the biogenesis of leader-derived siRNA. A facilitating role of the micro RNA (miRNA) biosynthetic enzyme DCL1 on accumulation of DCL2-, DCL3- and DCL4–dependent siRNAs derived from the 35S leader. Several leader-derived siRNAs showed near perfect sequence complementarity to *Arabidopsis* transcripts. By employing a sensor transgene, a direct evidence for at least one of those molecules acting as a bonafide siRNA in infected turnip, was obtained. More than 100 transcripts potentially targeted by CaMV-derived siRNAs were identified through extensive bioinformatics searches. Many of these transcripts were effectively downregulated during CaMV infection (Moissiard and Voinnet 2006).

RNA silencing is considered to represent an adaptive immune response of plants against plant viruses. In plants infected by RNA viruses, the presence of distinct siRNAs of distinct size classes have been observed. So also DNA viruses induce different size classes of siRNAs. RNA viruses are commonly targeted by DCL4 and DCL 2 which produce 21- and 22-nt viral siRNAs. DCL2 and DCL3 were implicated in the production of 22- and 24-nt geminiviral siRNA respectively and an additional DCL activity producing 21-nt geminiviral siRNAs (Akbergenov et al. 2006). Two nuclear DNA viruses, *Cabbage leaf curl virus* (*CaLCuV*) (*Geminivirus*) and *Cauliflower mosaic virus* (CaMV) (pararetrovirus) and a cytoplasmic RNA virus, *Oilseed rape mosaic virus* (ORMV, tobamovirus) are differentially targeted by subsets of DCLs. DNA virus–derived siRNAs (21, 22 and 24-nt) were produced by all four DCLs including DCL1 known to process microRNA precursors. DCL1 was found to specifically generate 21-nt siRNAs from the CaMV leader region. In contrast, ORMV (RNA virus) was mainly affected by DCL4. All the four DCLs were partially redundant for CaLCuV-induced mRNA degradation. On the other hand, DCL4 in conjunction with RNA-dependent RNA polymerase 6 (RDR6) and the methyl-transferase HEN1 specifically facilitated extensive virus-induced silencing in new tissues. Furthermore, CaMV was shown to impair processing of endogenous RDR6-derived ds-RNA, while ORMV prevented HEN1-mediated methylation of siRNA duplexes. The results suggested the operation of two novel viral strategies of silencing suppression (Blevins et al. 2006).

Posttranscriptional gene silencing (PTGS) functions as an endogenous defense mechanism against plant virus infection by directly targeting viral genome integrity and consequently lowering the titer of the invading virus. It is possible to redirect this mechanism to target indigenous host mRNAs by introducing corresponding plant cDNA fragments within the viral genome, thus providing a means to downregulate host genome expression (Kumagai et al. 1995). This approach designated virus–induced gene silencing (VIGS) permits rapid transient knockdown of host gene expression. VIGS takes advantage of the PTGS defense system to silence endogenous RNA sequences that are homologous to a sequence engineered into a viral genome which generated dsRNA that mediates silencing, whereas VIGS offers more flexibility. PTGS-based approaches need stable transformation of antisense or inverted repeat sequences (Wesley et al. 2001). Several RNA or DNA viruses with different specificities and host range have been demonstrated to be useful tools for development of potential VIGS vectors using *N. benthamiana* as a host

(Robertson 2004). RNA viruses TMV, PVX and *Tobacco rattle virus* (TRV) and DNA virus *Tomato golden mosaic virus* (TGMV) have been used as VIGS vectors. This approach has been shown to be useful for the functional characterization of host plant endogenes in a variety of organs and tissues. Many host plant genes have been successfully silenced in foliar tissue, meristematic tissue and potato tubers (Ratcliff et al. 2001; Peele et al. 2001; Faivre-Rampant et al. 2004).

VIGS efficiency depends primarily on the ability of the virus to invade the host and replicate to a sufficient level in the targeted tissue. Tobraviruses such as TRV are transmitted by nematodes and require a viral helper protein encoded by the RNA2-2b gene (Vassilakos et al. 2001; Vellios et al. 2002). A modified TRV vector retaining the helper protein 2b required for transmission of TRV was constructed. This TRV vector replicated extensively in whole plants including meristems and also triggered a pervasive systemic VIGS response in the roots of *N. benthamiana, Arabidopsis* and tomato. Roots and silenced plants exhibited reduced levels of target mRNA. The TRV-2b vector exhibited enhanced infectivity and meristem invasion, both key requirements for efficient VIGS based functional characterization of genes in root tissues. The results suggested that the TRV-2b helper protein might have crucial role in host regulatory mechanism capable of controlling TRV invasion (Valentine et al. 2004).

The distinct advantage of TRV-based VIGS in solanaceous plants is the ease of introduction of the VIGS vector into plants. This is usually mediated by *Agrobacterium tumefaciens* with the VIGS vector placed in between T-DNA borders (Ratcliff et al. 2001; Liu et al. 2002). *A. tumefaciens* can be easily introduced into plant tissues by different procedures. A protocol involving agroinfiltration of VIGS vector carrying fragments of genes of interest into seedlings at the 2 to 3-leaf stage and requiring minimal modification has been developed. This procedure is suitable for *N. benthamiana,* tomato and the model plant species *A. thaliana.* The results demonstrate that VIGS can be used to silence genes involved in general metabolism and development of disease resistance. This approach is also effective at knocking down expression of highly expressed transgenes (Burch-Smith et al. 2006).

Virus-induced gene silencing (VIGS) has been employed less frequently to analyze gene functions in monocotyledonous plants partially due to nonavailability of suitable virus expression vectors. A strain of *Brome mosaic virus* (F-BMV) infecting *Festuca arundinacea* (tall fescue) causes systemic symptoms in rice, barley and a cultivar of maize Va35 under greenhouse conditions. The genetic determinants controlling systemic infection of rice were mapped to RNAs 1 and 2 of the tripartite genome. Coinoculation of RNAs 1 and 2 from F-BMV and RNA3 from R-BMV (modified Russian strain to accept inserts from foreign genes) expressing a portion of a plant gene to leaves of barley, rice and maize plants produced visual silencing-like phenotypes. The BMV vectors may prove to be useful for analysis of gene function in rice and maize for which VIGS system is not available (Ding et al. 2006).

Many viruses can encode proteins that suppress PTGS at different points of its pathway, as a counter defensive strategy. The helper component HC-Pro of

some potyviruses has been shown to function as suppressors of PTGS (Brigneti et al. 1998). The HC-Pro was reported to interfere with initiation and maintenance steps of PTGS. HC-Pro of *Tobacco etch virus* (TEV) partially inhibited PTGS induced by a transiently expressed dsRNA from a jelly fish GFP at early stages of PTGS (Johansen and Carrington 2001). The PTGS-deficient mutants of *Arabidopsis* became hyper-susceptible to infection by *Cucumber mosaic virus* (CMV), leading to excessive accumulation of CMV RNA (Vance and Vaucheret 2001). The potyvirus *Turnip mosaic virus* (TuMV) entirely inhibited PTGS in *Arabidopsis,* whereas CMV only partially inhibited PTGS (Mourrain et al. 2000). The viruses produce specific proteins that are involved in the inhibition of PTGS. In the case of HC-Pro protein of potyviruses, PTGS inhibition appears to result from the activation of the cellular *rgs–CaM* gene (Anandalakshmi et al. 2000). The p25 protein of potexviruses was presumed to be involved in the inhibition of PTGS and inhibition of the propagation of the systemic silencing signal of PTGS (Voinnet et al. 2000). The product of expression of ORF2 of *Potato virus X* (PVX), the p25 was considered to interfere with the assembly of si-RNAs in RISC (Voinnet et al. 2000). On the other hand, tombusvirus p19 was able to bind with siRNAs, thus making them unavailable for processing by RISC (Lakatos et al. 2004). These proteins have been shown to interfere with the steady state levels of miRNAs (Chapman et al. 2004; Chellappan et al. 2005).

A novel RNAi suppression mechanism by *Red clover necrotic mosaic virus* (RCNMV) has been recognized. Multiple viral components including viral RNAs and putative RNA replicase proteins are required to suppress generation of RNAi. A close relationship between RNA elements required four negative strand RNA synthesis and RNAi suppression suggested a strong link between the viral RNA replication machinery and RNAi machinery. In a transient assay, RCNMV interfered with the accumulation of siRNA in RNAi – induced by a hairpin dsRNA and it also interfered with micro RNA (miRNA) biosynthesis. The results suggested that RCNMV for its replication, deprives the RNAi machinery of DICER – like enzymes that are involved in both siRNA and miRNA biosynthesis (Takeda et al. 2005).

The *Grapevine virus A* (GVA) genome was screened for the presence of a gene potentially capable of suppressing RNA silencing. Four of the five proteins encoded by GVA genome was screened using a GFP based transient expression assay. The expression product of ORF5 (protein p10) had the property of suppressing silencing induced by a transiently expressed ss-sense RNA. GVA p10 was found to be a suppressor belonging to a new family. This protein reduced the levels of siRNAs markedly and the recombinant protein bound ss- and ds-forms of siRNAs and microRNAs, suggesting the existence of a potential mechanism of suppression based on RNA sequestering (Zhou et al. 2006b).

Plant viruses belonging to *Tombusviridae* have icosahedral particles with linear, small positive sense ss-RNA genomes. Distinct suppressors of RNA silencing are produced by viruses belonging to different genera. The CP of *Turnip crinkle virus* (genus *Carmovirus*) has multiple functions – formation of capsid and suppression

of silencing. In contrast, a 19-kDa suppressor protein (P19) is required only for inhibition of silencing in the case of *Tomato bushy stunt virus* (TBSV) (Qu and Morris 2002). This suppressor protein binds ds-RNA size selectively and forms strong complexes with ds-RNAs having 19-nt duplex regions both in vitro and in vivo. In tombusvirus – infected cells, P19 may sequester silencing – generated siRNAs, resulting in suppression of antiviral silencing responses (Lakatos et al. 2004). *Pothos latent virus* (PoLV) (*Aureusvirus*, genus) produces a 14-kDa protein which has been shown to be an efficient suppressor of both virus- and transgene-induced silencing. P14 prevented the accumulation of hairpin transcript-derived siRNAs, indicating that P14 could inhibit inverted repeat-induced silencing by binding the long ds-RNA precursors of siRNAs. However, accumulation of viral siRNAs to high levels could be observed in PoLV-infected plants. This may possibly be due to sequestering of ds-siRNA by P14 protein leading to inhibition of virus-induced silencing (Mérai et al. 2005).

Viroids and viral satellites have been shown to have small, noncoding and highly structured RNA genomes. The ability of both viroids and viral satellites to induce RNA silencing, like the viruses, has been demonstrated in some pathosystems. In order to determine whether *Potato spindle tuber viroid* (PSTVd) could trigger the production of small RNAs in infected tomato plants were analyzed for the presence of small RNAs with sequence specificity to PSTVd. Small RNAs of approximately 25-nt were detected in both mild strain- and RG1-infected tomato plants, but not in mock-inoculated plants. The siRNAs exhibited sequence specificity to PSTVd, indicating the operation of PSTVd–induced RNA silencing (VdIRS). However, RNA silencing did not seem to be responsible for variation in symptoms induced by the mild and severe strains (RG1) of PSTVd (Itaya et al. 2001). In another investigation, the siRNAs induced following infection of tomato by PSTVd, were of 22- and 23-nt representing different domains of the viroid genome. The presence of these siRNAs indicated that the nuclear replicating PSTVd RNA could induce PTGS. The siRNAs were slightly more abundant at 30 dpi than at later stages. No apparent relationship was noted between the titers of PSTVd-specific siRNAs and the titers of viroid strain (Papaefthiniou et al. 2001).

Tomato plants expressing harpin RNA derived from PSTVd developed symptoms similar to those of PSTVd infection. Further, if RNA silencing in tobacco was prevented using a silencing suppressor, symptoms caused by *Cucumber mosaic virus* (CMV) satellite Y were markedly reduced. These results suggest that viroids and satellites appear to induce disease symptoms by directing RNA silencing against physiologically important host genes. Viroid and satellite RNAs exhibit remarkable resistance to RNA silencing-mediated degradation that is effective against viruses. Most viroids including PSTVd replicate in the nucleus and this subcellular localization possibly protect them from RISC believed to be functioning in the cytoplasm. Nevertheless, viroids have to traverse the cytoplasm during cell-to-cell movement, during which time, they are likely to be exposed to the cytoplasmic RISC degradation. Probably the extensive intramolecular base pairing may render them less accessible to the RISC complex for degradation (Wang et al. 2004).

Appendix: Development of Sequence-Tagged Site (STS) Marker Linked to Bacterial Wilt Resistance Gene (Onozaki et al. 2004)

A. Random Amplified Polymorphic DNA (RAPD) Analysis

i. Harvest young partly expanded leaves of 2–3 month old carnation plants; wash thoroughly with DW; and extract total DNA from 0.5 g of leaf tissue samples as per cetyltrimethyl ammonium bromide (CTAB) procedure.
ii. Use 465 random 12-mer primers (Wako Life Science, Osaka, Japan) and 40 random 10-mer primers (Operon Technologies Inc., Alameda California, USA).
iii. Perform PCR in a 10-µl reaction solution containing 1 x EX Taq buffer, 20 ng of total DNA, 20 µM of each dNTP, 3 pmol of primer, and 0.25 units. Ex Taq DNA polymerase (Takara Biomedicals, Tokyo, Japan); amplify in a Takara PCR Thermal Cycler MP (Takara Biomedicals).
iv. Adopt the following temperature schedule: 94°C for 30 s; 45 cycles at 94°C for 30 s, 40°C for 1 min 30 s; 72°C for 2 min 30 s; and 72°C for 7 min; followed by cooling at 4°C to stop the reaction.
v. Analyze the amplified products through electrophoresis at 100 V for 40 min in 1.5% agarose gels (type I-A, Sigma, Steinheim, Germany) containing ethidium bromide in 0.5 x TBE buffer; photograph under UV light on Polaroid film and score for the presence of a particular polymorphism.

B. Bulked Segregant Analysis (BSA)

i. Prepare two different pooled DNA samples from equal volumes of standardized DNA from five resistant and five susceptible selected plants from progeny plants; use predetermined number of primers (505 in this study) simultaneously to screen the resistant and susceptible pooled DNAs and two parents.
ii. Select the primers that can detect different marker patterns between the pooled DNAs and between the two parents and test these primers on the same five individual resistant and five individual susceptible plants.
iii. Run the selected markers (eight) on the whole population of all individuals (134) and construct a partial linkage map using MAPMAKER/EXP version 3.00 program.

C. Conversion of RAPD Marker into STS Marker

i. Cut out the agarose gel block containing the RAPD marker band; purify with a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

ii. Ligate the purified DNA to the plasmid vector PT7 Blue T (Novagen Inc., La Jolla, California, USA) using a DNA ligation Kit (Takara Biomedicals) and transform into *Escherichia coli* strain JM109.
iii. Select 10 white colonies, after transformation, on X gal + IPTG LB agar plates (ampicillin 100 µg/ml) and culture them separately overnight on the same type of agar plate.
iv. Use an aliquot of each colony as template for PCR using RAPD primer for the target RAPD marker band.
v. Confirm the presence of the insert concerned by digesting the PCR products with *TaqI* restriction enzyme and then compare the fragment size with the eluted polymorphic band.
vi. Select the colony positive for the insert and grow in a large culture.
vii. Isolate the plasmid DNA and purify using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, California, USA).
viii. Sequence the cloned fragments using a PRISM377DNA sequencer (Applied Biosystems, Foster City, California, USA).
ix. Design STS primer sets of about 20-mer oligonucleotide using the primer design program Primer 3 on the basis of the sequence data and perform PCR with the STS primers in a 10-µl reaction solution containing 1x EX Taq buffer, 20 ng of total DNA, 200 µM of each dNTP, 1.5 pmol of each forward and reverse primer and 0.25 units ExTaq DNA polymerase.
x. Amplify using a Takara PCR Thermal Cycler MP; use a temperature schedule as follows: 94°C for 30 s; 30 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 30 s and 72°C for 5 min followed by cooling at 4°C to stop the reaction.
xi. Follow steps as in A (v) above

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Chapter 4
Transgenic Resistance to Crop Diseases

Abstract It is well recognized that the use of cultivars resistant to diseases caused by microbial plant pathogens is the most important disease management strategy. However, the difficulties associated with the conventional breeding methods, necessitated the development of alternative methods of obtaining disease resistant cultivars. Genetic engineering approach has been demonstrated to provide enormous options for the selection of the resistance genes from plants, microbes, insects, animals and pathogens themselves. The molecular markers have been successfully applied to locate, clone and characterize resistance genes. The expression of transgenes in transgenic plants can be verified by using appropriate molecular techniques. Among several approaches made, pathogen-derived resistance has been widely tested and found to be practicable as in the case of *Papaya ring spot virus* disease. In the case of bacterial and fungal diseases, the potential of transgenic plants expressing antifungal and antibacterial proteins has been indicated by many studies. However, the acceptance of products of genetically modified crops by the consumers remains an unanswered question.

Management of crop diseases caused by microbial pathogens by incorporating genes conferring disease resistance from different sources has been discussed earlier (Volume 3, Chapter 3). Molecular biology of pathogenesis and disease resistance has been intensively researched facilitating a better understanding of the functions of virulence and avirulence genes of the microbial pathogens and activation of a series of host responses resulting in enhancement of resistance to pathogens. Genetic engineering techniques provide enormous options for the selection of the desired gene(s) from a wide range of sources not only in the plant kingdom but also in the animal kingdom and even from the pathogens themselves. The disease resistance genes present in various sources, especially wild relatives of crop species have been located at specific sites by the action of restriction endonucleases. The DNA fragment(s) carrying the desired gene(s) are identified and used as molecular markers. Random amplified polymorphic DNA (RAPD) technique in conjunction with PCR assay has been employed to identify resistance genes rapidly. These genes are then cloned in test organisms to study the nature of gene products and gene action. The PCR assay has been demonstrated to be useful to amplify the specific sequences in the DNA.

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Transgenic plants constitutively expressing defense-related genes from desired sources can be generated. The exogenous DNA, introduced artificially via genetic engineering techniques, has to pass through the germ line of the plant to be transformed, following incorporation of the foreign DNA into the plant's nuclear material. New crop varieties with resistance to diseases may be developed within a short period by employing the various biotechnological methods. On the other hand, classical breeding procedures require several years, in addition to the obstacles posed by interspecific sterility and linkage to undesirable traits. Furthermore, multiplex, diverse genes conferring resistance to diseases may be incorporated simultaneously by transgenic plant technology, which permits a wider genetic diversity to be exploited. Transformation of plants by transferring desirable genes from bacteria, fungi, viruses or various species of plants has been achieved mostly by employing the phytopathogenic bacterium *Agrobacterium tumefaciens* (*At*). The Ti-(tumor inducing) plasmid of *At* containing T-DNA has been used for gene transfer to plants. The T-DNA can be disarmed, rendering the plasmid nononcogenic, but still retaining its ability to integrate with plant host genome and to synthesize opines. It has been demonstrated that the T-DNA can be trimmed down to contain only absolutely essential functions for providing more room for cloning exogenous DNA fragment. Depending on the nature of pathogens and their pathogenic potential (virulence), different techniques have been applied to obtain crop cultivars with enhanced resistance to diseases.

4.1 Resistance to Virus Diseases

Sources of resistance to crop virus diseases may be primarily of three classes viz., (i) genes derived from viral sequences providing pathogen-derived resistance (PDR); (ii) genes from various other sources that can interfere with target virus and (iii) natural resistance genes. The sources of natural resistance genes and the molecular biology of resistance provided by them have been discussed in (Volume 3, Chapter 3).

4.1.1 Pathogen-Derived Resistance

The transgenic expression of pathogen sequences was postulated to provide protection against the respective pathogen itself by interfering with its development or multiplication (Sanford and Johnson 1985). Currently the term pathogen-derived resistance (PDR) has been generally accepted, although several other terms were earlier applied. This is the principal approach by which transgenic protection is offered against many viruses affecting a wide range of crop plants with varying degrees of success and acceptability. Powell-Abel et al. (1986) demonstrated that incorporation of coat protein (CP) gene of *Tobacco mosaic virus* (TMV) in tobacco protected them against TMV. The pathogen-derived gene interferes with the replication

process of viruses in their host plants in different ways. The gene product expressed in the transformed plant may inhibit the normal function(s) of the cognate gene in the infecting viral genome or the gene product may be so modified as to become detrimental to viral replication or maturation. The genomes of several plant viruses have been well characterized. The functions encoded by different viral genes have also been determined. Hence, it has been possible to transform plants with different viral genes encoding structural and nonstructural proteins and the effectiveness of protection provided against viruses has been assessed.

4.1.1.1 Protection by Viral Coat Protein (CP) Genes

Use of CP genes to protect plants against viral infection has been the most common strategy applied based on PDR genes against viruses. The possibility of reducing the effects of virus infection has been indicated in the case of large number of host-virus combinations (Narayanasamy 2002). The transgenic resistance due to expression of CP gene in tobacco plants has certain specific characteristics. A cloned DNA encoding the CP gene of TMV was ligated to the *Cauliflower mosaic virus* (CaMV) 35S promoter and NOS3′ (nopaline synthase gene 3′ end) polyadenylation signal. This chimeric gene was introduced into tobacco cells on a disarmed Ti-plasmid of *A. tumefaciens*. The transgenic tobacco expressing CP gene either did not develop symptoms or developed symptoms after a longer incubation period. The virus titre was very low or TMV could not be detected in some plants (Powell-Abel et al. 1986; Beachy et al. 1990). The resistance of transgenic tobacco plants was only against intact virus, but not against viral RNA, since inoculation of transgenic plants with TMV RNA resulted in production of symptoms in as many plants as in nontransgenic control plants (Nelson et al. 1997). Transgenic tobacco plants expressing CPs of TMV or *Soybean mosaic virus* (SMV) exhibited resistance to tobamoviruses and potyviruses respectively (Nejidat and Beachy 1990; Lawson et al. 1990). Transgenic plants expressing the *CP* gene of *Potato leaf roll virus* (PLRV) exhibited resistance to PLRV, when inoculated by viruliferous aphids (Kawchuk et al. 1990) indicating the usefulness of *CP*-mediated resistance under field conditions. Similar results were reported in the case of rice-*Rice stripe virus* (RSV) pathosystem also (Hayakawa et al. 1992).

The ability of *CP* gene of plant viruses functioning as an elicitor of resistance was indicated by de la Cruz et al. (1997). In capsicum, the *L*2 gene confers resistance to all known tobamoviruses except *Pepper mild mottle virus* (PMMoV). A hybrid virus consisting of the *CP* gene of *Paprika mild mottle virus* (Pa MMV) which can induce a HR and the PMMoV-S sequences was able to elicit HR in *Capsicum frutescens* (L^2L^2) plants, suggesting that the CP gene has the sequences that affect induction of HR-mediated L^2 resistance in pepper. The CP, but not the RNA appears to be the elicitor of HR in pepper. In eggplant, specific tobamovirus CPs have been reported to act as elicitors of HR. Eggplants inoculated with TMV developed HR, but not when inoculated wih a TMV mutant unable to express the *CP* gene. The CPs of U2 strain of TMV and *Odontoglossum* ring spot tobamovirus also elicited HR, whereas the *CP* gene of cucumber mosaic tobamovirus did not elicit HR in

eggplant indicating that the elicitation of HR may be associated with *CPs* of specific tobamoviruses (Dardick and Culver 1997). The potato cv. Igor was nearly ruined by NTN isolates of *Potato virus Y* (PVY^{NTN}) causing potato necrotic ringspot disease (PTNRD). This cultivar was transformed with the *CP* gene of PVY^{NTN} and several transgenic lines were highly resistant, showing no visible symptom, when they were graft-inoculated. The virus could not be detected by either ELISA or infectivity test. Among the 34 transgenic lines, two showed resistance to PVY^{NTN} and PVY^{O} and one line was resistant to PVY^{O} (Racman et al. 2001).

Papaya ringspot virus (PRSV), causing serious losses in several countries, exists in the form of different strains. Transgenic papaya plants expressing the CP gene of PRSV have been demonstrated to be highly resistant not only in the greenhouse conditions but also under field conditions. The transformed plants were not infected, whereas 91 to 100% of the nontransformed plants were infected, indicating the effectiveness of transgenic resistance provided by *CP* gene of PRSV to the cultivars SunUp and Rainbow, homozygous and hemizygous for the *CP* transgene respectively. Growing cultivars with tansgenes that can provide effective protection against virus diseases can be a profitable disease management approach (Ferreira et al. 2002). Four transgenic papaya lines expressing the *CP* gene of PRSV were evaluated for their usefulness under field conditions, in another investigations. At 3 to 5 months after planting some of the transformed papaya plants exhibited mild symptoms of PRSV infection, whereas nontransformed plants showed severe disease symptoms. However, the fruit yield and quality of the fruits were not affected in transgenic plants which recorded significant increases in the yield (10 to 56%) over the nontransformed papaya plants (Bau et al. 2004). In another investigation, four constructs of *CP* genes of PRSV, in the sense orientation (S-CP), antisense orientation (As-CP), sense orientation with a frame shift mutation (FS-CP) and sense orientation with three-in-frame stop codons (SC-CP) were used to transform papaya via *A. tumefaciens*-mediated transformation. Highly resistant were marked in each group, after inoculating the transgenic plants mechanically with PRSV H1K strain. Crosses were made between 21 PRSV resistant lines representing the four transgene constructs and six papaya genotypes. The lines from the FS-CP and SC-CP transgenic groups were highly fertile, whereas the lines belonging to other two groups were practically infertile. In the field evaluation, the R_1 progenies of transformed lines showed 23.3% natural infection, whereas non-transformed plants had high level of infection (96.7%) after one year, indicating the possibility of selecting PRSV-resistant plants by combining the transgenic resistance strategy with conventional breeding procedure (Davis and Ying 2004).

The transgenic papaya varieties Rainbow and SunUp carrying CP gene released commercially as resistant to PRSV were derived from the line 55-1 by conventional breeding methods. SunUp is homozygous for the single CP transgene insertion, while Rainbow is a F_1 hybrid between SunUP and the cv. Kapoho Solo and hemizygous for the *CP* transgene. Resistance to PRSV was demonstrated to be RNA-mediated via post-transcriptional gene silencing (PTGS) and dependent on plant age and gene dosage (Tennant et al. 2001). Another line 63-1 was derived from Sunset, transgenic papaya expressing the CP gene from a mild mutant of a Hawaiian isolate of PRSV. These plants showed a range of resistance to severe PRSV isolates from

Hawaii (HA), Jamaica (JA), Thailand (TH) and Brazil (BR). Genetic and molecular analyses confirmed that the line 63-1 had two *CP* transgene insertion sites and the *CP* and *nptII* genes are present at both loci. The number of resistant plants in a 63-1 derived population was directly correlated with the number of plants with multiple transgene copies. The results indicated that transgene dosage had a pivotal role in determining the degree of resistance of the line 63-1 to PRSV isolates from different ecosystems (Souza Jr et al. 2005).

Plum pox virus (PPV) causes an important disease (sharka) in commercial stone fruit (*Prunus* spp.) including plum-apricot and peach. Transgenic clones of *Prunus domestica* were produced via *A. tumefaciens* transformation of plum hypocotyles with *CP* gene of PPV (Scorza et al. 1994) and one clone C5 exhibited high level of resistance to PPV under glasshouse conditions (Ravelonandro et al. 1997). In a later study, transgenic clones C2, C3, C4, C5, C6 and PT-6 expressing CP gene of PPV and PT-23 transformed with marker genes only and a nontransgenic line were evaluated for sharka disease resistance in Poland and Spain under field conditions where disease pressure was high for 7 years. Only clone C5 exhibited high resistance. None of the C5 trees became naturally infected, while upto 100% of other plum trees showed disease symptoms and tested positively for ELISA and RT-PCR techniques. The C5 clone showing distinct posttranscriptional gene silencing (PTGS) (Volume 3, Chapter 3) remained healthy for at least 7 years under field conditions, despite the high disease pressure representing a significant part of the productive life of plum orchards (Malinowski et al. 2006).

Passion fruit woodiness virus (PWV *Potyvirus*) causes an economically important disease in Brazil. A full length *CP* gene from a severe PWV isolate was cloned into pCAMBIA 2300 binary vector which was used for transformation via *A. tumefaciens* strain EHA105. Leaf disks were used as explants for transformation and integration of PWV CP was confirmed in seven of eight plants regenerated, using Southern blot analysis. Different numbers of insertional events for the *CP* gene were observed. Although clones of all transgene plants exhibited some resistance to PWV infection, T2 plants were highly resistant even at high disease pressure. These transgene plants showed resistance to all three PWV isolates tested (Trevisan et al. 2006). In an earlier investigation, transgenic plants expressing the 3′ region of the *Nib* gene and the 5′ region of the *CP* gene from *Cowpea aphid borne mosaic virus* (CABMV) provided protection to only one isolate of PWV (Alfenas et al. 2005). The highly resistant or immune transgenic passion fruit plants (T2) had three insertional events of the transgene. The presence of multiple copies of a transgene has been shown to be associated with RNA-mediated resistance which has been linked to immunity and high levels of resistance to infection (Lindbo and Dougherty 2005). *CP* gene expression might have been either suppressed or silenced in the T2 transgenic passion fruit plants, since transcribed RNA could not be detected by Northern and Western blotting, suggesting the operation of a mechanism involving RNAi for the high level of resistance or immunity of T2 plants to all three isolates of PWV (Trevisan et al. 2006).

The CP-mediated resistance to viruses affecting various plant species has been demonstrated to offer effective protection. The mechanism of CP-mediated resistance (CP-MR) seems to vary, depending on the host-virus combinations. In the

case of TMV, CP interferes with the disassembly of virus particles and hence CP-MR can be overcome when plants are inoculated with TMV-RNA (Register and Beachy 1988). In contrast, transgenic potato plants expressing PVX CP, were resistant to both the intact virion and its RNA. It was hypothesized that PVX CP may either interact with origin of assembly and restrict replication or interfere with translation of the replicase gene (Hemmenway et al. 1988; Spillane et al. 1997). In order to determine the possibility of PTGS having a role in the development of CP-MR against PVX infection, transgenic tobacco plants expressing PVX CP were challenged with PVX under conditions in which PTGS was suppressed by low temperatures or by using PVY that encoded HC-Pro, a known suppressor of PTGS. The transgenic tobacco plants expressing PVX CP, were maintained at 15 and 24°C for 10 days. Plants were inoculated by particle bombardment with cDNA of the virus control of the CaMV 35S promoter. The transgenic plants were resistant producing very few small sites of infection as against production of high number of sites of infection in nontransformed control. The results showed that PVX CP-MR was not temperature dependent, indicating resistance was not dependent on PTGS. The transgenic plants when inoculated with PVY exhibited symptoms of PVY comparable to those plants infected by a single virus, suggesting that PTGS may not have a significant role in the PVX-CP-MR (Bazzini et al. 2006).

Grapevine fan leaf virus (GFLV, *Nepovirus*) a nematode-transmitted virus has three particle types the T (top), M (middle) and B (bottom) which occupy the sequential positions in a purified viral suspension because of the particle density. The T component contains empty virus capsids; while M and B components have viral RNA2 and RNA1 + RNA2 respectively. The viral CP, encoded by RNA2 determines vector specific transmission by *Xiphimema index*, in addition to encoding RNAs. Putatively transgenic individuals (127) of *Vitis vinifera* cv. Russalka were generated by transferring the CP gene including nontranslatable and truncated forms via *A. tumefaciens*-mediated transformation. The transgenic sequences were detected by PCR in all lines. In addition, Southern blot analysis revealed the number of inserted T-DNA copies varying from 1 to 6. Accumulation of GFLV-CP could not be detected by ELISA in any one of the 36 transgenic lines analyzed. However, RT-PCR analysis indicated that the GFLV CP mRNA was expressed at variable levels. It is argued that absence of CP in detectable concentration might be an advantage from a safety point of view. However, the level of resistance/tolerance of the transgenic lines of grapevine to GFLV remains to be determined. Hence, the potential value of these lines for practical utility can not be adjudged (Maghuly et al. 2006).

As the sources of high resistance to *Lettuce big-vein associated virus* (LBVaV) disease as yet not found, the possibility of developing transgenic plants expressing CP gene of LBVaV was explored. The CP gene in sense or antisense orientation in a binary vector pBI121 was used to transform lettuce plants via *Agrobacterium tumefaciens*-mediated transformation. The presence of the CP gene in transformed plants was confirmed by PCR assay. Southern blot analysis showed that one copy of the LBVaV CP gene and one copy of the *nptII* gene were present in the transformed plants. The transgenic A-2 line with CP gene in antisense orientation was resistant to LBVaV. This line was also resistant to the *Mirafiori lettuce virus* (MiLV) (Kawazu et al. 2006).

4.1.1.2 Protection by Noncoat Viral Genes

Attempts have been made to assess the effectiveness of transformation of crop plants with viral genes other than coat protein gene, in providing protection against virus diseases. Transgenic tobacco plants expressing the 54 K replicase protein of the U1 strain of TMV were highly resistant to the same strain and the effectiveness of protection was better than that provided by *CP* gene of TMV. The resistance was effective against both TMV virions and TMV RNA, whereas the CP-mediated resistance was effective against TMV virions only (Golemboski et al. 1990). Likewise, the transgenic tobacco expressing either the intact ORF1 gene or the 5′ half of *Potato virus X* (PVX) exhibited high levels of resistance against PVX infection, being approximately 10-folds greater than that obtained with CP-gene-mediated resistance (Braun and Hemenway 1992). Putative replicase-mediated resistance against *Pea early browning virus* (PEBV, *Tobravirus*) has also been demonstrated. The transgenic plants were resistant to PEBV and its strains and another tobravirus *Broad bean yellow band virus* also (MacFarlane and Davies 1992). In tomato lines transformed with a defective replicase gene from RNA2 of *Cucumber mosaic virus* (CMV), the virus could not be detected in uninoculated leaves, though a low concentration of CMV was present initially in the inoculated leaves. The transgenic plants were resistant to many strains of CMV. The long-distance movement of CMV appeared to be affected by the replicase-mediated resistance, because of the virus was unable to move from the infected root stock of nontransformed tomato or tobacco into transgenic scions (Gal-On et al. 1998).

The ability of the sense and antisense RNA of the replication-associated protein encoded by *AC1* or *C1* gene of geminiviruses to protect plants against virus infection was assessed. Transient expression of *AC1* or the truncated N-terminal portion of the protein reduced the viral DNA replication in tobacco protoplasts. *Nicotiana benthamiana* plants expressing the AC1 coding sequence, when challenged, either were symptomatic or produced attenuated or delayed symptoms. Virus titres (DNA) in transgenic plants were significantly reduced. The resistance was specifically against *African cassava mosaic virus* (ACMV) and the transgenic plants were not resistant to other geminiviruses tested (Hong and Stanley 1996). Resistance to *Tomato yellow leaf curl virus* (TYLCV) could be incorporated by transforming tomato plants with a truncated version of the *C1* gene of TYLCV encoding the first 210 amino acids of the multifunctional Rep protein. One transformant (RO) carrying the *C1* gene in two loci (A and B) and accumulating the truncated Rep protein (T-Rep) was crossed with a wild plant. The progenies containing either A or B locus were homogeneous, expressed high level of T-Rep protein and were resistant to TYLCV, when inoculated by viruliferous whiteflies or by agroinfection. The accumulation of T-Rep protein is essential for resistance to TYLCV (Brunetti et al. 1997).

Cucumber fruit mottle mosaic virus (CFMMV, *Tobamovirus*) is soilborne and causes severe wilting, in adidtion to mosaic and yellow mottling on leaves and fruits. The gene coding for the putative 54-kDa replicase gene of CFMMV was cloned into *A. tumefaciens* binary vector and cotyledons as explants of a parthenocarpic cucumber were transformed. Of the total 14 lines containing replicase, eight

lines showed high level of resistance to CFMMV and both biological and molecular (RT-PCR) methods did not show the presence of the virus. The line I 44 was found to be resistant by graft-inoculation and it was homozygous for the putative 54-kDa replicase gene. Furthermore, the line was resistant, when plants were planted in CFMMV-infested soil. Symptom appearance was significantly delayed, when the line I 44 plants were infected by three additional cucumber-infecting tobamoviruses viz., *Cucumber green mottle mosaic virus* (CGMMV-W), *Kyuri green mottle mosaic virus* (KGMMV) and *Zucchini green mottle mosaic virus* (ZGMMV). When used as a root stock, the line I 44 could protect susceptible cucumber scions from soil infection by CFMMV. The accumulation level of the 54-kDa locus transcript in the line I 44 was consistently low in spite of being driven by ΔSV-a strong constitutive promoter. Inoculation of I 44 plants with CFMMV or ZYMV did not alter the RNA expression level of the 54 kDa transgene. This investigation indicated that it would be possible to protect the susceptible scions against soilborne viruses, by grafting them on transgenic root stocks (Gal-On et al. 2005).

The effectiveness of movement protein (MP) genes in protecting transgenic plants has been found to be variable. Tobacco plants expressing 30 K MP gene of TMV did not show any decrease in their susceptibility to TMV infection (Deom et al. 1987). Strangely, transgenic tobacco Xanthi D8 NN plants expressing the p24 protein implicated in local movement *Potato virus X* were resistant to TMV as revealed by the production of smaller necrotic local lesions and also to another tobamovirus Ob, that evades the HR provided by the *N* gene (Ares et al. 1998). In addition, tobacco plants expressing a mutated form of the MP gene *BC1* of *Tomato mottle* virus (ToMV, *Geminivirus*) showed resistance to ToMV and also to another geminivirus *Cabbage leaf curl virus* (CabLCV). The presence of ToMV was detected in the inoculated leaves, but not in leaves away from inoculated sites in the highly resistant plants (Duan et al. 1997). In certain pathosystems, transgenic expression of viral gene may cause undesirable effects on the host plant development. For example, the expression of MP of *Bean dwarf mosaic virus* (BDMV) adversely affected the development of tomato plants (Hou et al. 2000).

The possibility of producing transgenic plants with broad-spectrum resistance was investigated by introducing tandemly cloned viral *N* gene sequences of tospoviruses. Transgenic tobacco plants expressing nucleoproteins (N) of three different tospoviruses, viz. *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) were generated. Transformed tobacco plants exhibited resistance to all three tospoviruses (Prins et al. 1995). In a further study, tobacco plants were transformed with a wide range of randomly selected sequences of TSWV-RNA genome or its complements for their resistance to TSWV. The transgenic tobacco plants expressing *N* or *NSm* gene sequences only exhibited resistance to TSWV (Prins et al. 1996).

Potyviruses form a major group of plant viruses with genome in the form of a single large ORF encoding a large primary polyprotein. The virus-encoded proteins are produced by self-proteolysis due to the action of the products of three viral cistrons. The nuclear inclusion body is composed of two virus-encoded proteins namely NIa and NIb. The *Potato virus Y* (PVY) RNA fragment containing the

cistron encoding PVY protease NIa was used to transform tobacco plants. Several of the transgenic plants expressing NIa were resistant to PVY infection and no PVY could be detected in these plants. Tobacco plants transformed with NIa-NIb and CP cistrons also exhibited resistance to PVY (Vardi et al. 1993). Likewise, transformation of tobacco with *NIa* gene of *Tobacco vein mottling virus* (TVMV) protected the Tobacco plants against TVMV infection. Following inoculation with TVMV, the transformed tobacco plants did not show any symptoms and the viroid did not accumulate in these plants. In contrast, initial symptoms of infection by *Tobacco etch virus* (TEV) and PVY were observed in tobacco plants transformed with *NIa* genes and these plants recovered. The *NIa* genes and these plants recovered. The *NIa* genes may provide different levels of protection depending on the virus concerned (Fellers et al. 1998).

The genome of *Tomato spotted wilt virus* (TSWV) contains three linear negative strand RNAs including L RNA (8897 nt), MRNA (4821 nt) and S RNA (2961 nt). The viral genome is enclosed in the nucleocapsid (N) protein and the virus particles are enveloped by a lipid membrane containing glycoproteins. The nucleocapsid protein (N) gene of TSWV Hawaiin L isolate in a sense orientation genes was used to transform peanut (groundnut) cv. New Mexico Valencia via *A. tumefaciens*-mediated transformation. RNA expression and production of *N* protein in transgenic peanut were observed. Progeny of transgenic peanut plants expressing *N* gene showed a 10 to 15-day delay in symptom expression following mechanical inoculation with TSWV isolate. All transgenic plants were protected by transgene *N* against systemic infection by TSWV (Li et al. 1997).

The transgenic tomato line 30-4 was generated by incorporating TSWV nucleoprotein (*N*) gene via *Agrobacterium*- transformation. This line showed complete resistance to TSWV (Vaira et al. 1995). Molecular analysis of this line showed the presence of single transgene insertion locus containing at least three copies of rearranged *N* gene sequences. TSWV N-specific aberrant transcripts, but not viral protein, were detectable. The tomato hybrids obtained from homozygous progeny of line 30-4 engineered for TSWV resistance were tested under field conditions in two locations along with their corresponding nontransgenic hybrids. None of the hybrid plants were infected by TSWV, whereas nontransgenic hybrid plants showed infection upto 50%. The seeds were tested to determine the extent of flow of transgene via pollen. No transgene flow could be detected in the protected crops. Immunity to TSWV infection was confirmed by mechanical inoculation in vitro and also by grafting and thrips vector. TSWV replication was inhibited at the primary infection site in the leaf discs inoculated with viruliferous thrips. The TSWV strains that could overcome resistance in *SW-5* tomato cultivars did not infect the transgenic tomato line 30-4. The presence of the transgene in hemizygous state seemed to be as efficient as homozygous state in preventing TSWV infection. In addition, the presence of 30-4 transgene did not affect any agronomic traits of tomato plants. This study indicated that the engineered resistance has a practical value of breeding cultivars of commercial interest, since it can be combined with naturally occurring resistance, leading to reduction in risk for development of resistance-breaking strains of TSWV (Accotto et al. 2005).

4.1.1.3 Protection by Viral Nucleic Acid-Based Systems

The complementary RNA molecules may bind to the RNA transcripts of specific genes resulting in blockage of their translation, these RNAs are termed as antisense or mic RNAs (messenger-RNA-interfering complementary RNAs). Tobacco plants expressing antisense RNAs for three regions of the *Cucumber mosaic virus* (CMV) genome were produced. One of these antisense RNAs protected the transgenic plants to some extent (Rezaian et al. 1988). The effectiveness of protection offered by antisense RNA was of a low order and also the plants transformed with RNA sequences complementary to the CP gene of CMV exhibited low level of resistance. The plants with antisense RNA sequences could inhibit symptom development and virus accumulation in plants inoculated with low inoculum concentration of CMV. On the other hand, the protection provided by the sense CP sequence was not affected by inoculum concentrations within the range of testing (Cuzzo et al. 1988). Likewise, tobacco plants expressing the TMV-CP gene were more strongly protected than plants with RNA sequences complementary to the *CP* gene. However, in some pathosystems encouraging results have been obtained. The sense and antisense versions of *CP* genes of *Bean Yellow mosaic virus* and *Potato lef roll virus* were found to be equally effective in protecting the transgenic plants (Kawchuk et al. 1990; Hammond and Kamo 1993). The antisense RNA complementary to the messenger RNA of the Rep protein encoded by the *C*1 gene of *Tomato yellow leaf curl virus* (TYLCV) offered effective protection to transgenic *N. benthamiana* plants and the replication of challenge TYLCV was completely suppressed. Transgenes mediating resistance to TYLCV protected the plants through at least two generations (Bendahmane and Gronenborn 1997).

The ds-RNAs or self-complementary hairpin RNAs (hpRNAs) that are formed during the intermediate steps of viral genome replication have been found to be key triggers of the RNA degradation mechanism (Bass 2000). Use of ds RNAs or hpRNAs as inducer of RNA-mediated resistance may be expected to be much more effective than simple sense or antisense constructs (Smith et al. 2000; Wang et al. 2000). The silencing effect induced by hpRNA constructs was demonstrated to be spectacular in protecting the host plants against PVY and *Barley yellow dwarf virus* (BYDV) (Smith et al. 2000; Wang et al. 2000; Wesley et al. 2001). Induction of silencing was of high order when inverted repeat constructs were used. Repeated viral transgene sequences provided high resistance frequencies even in (heterozygous) R_0 plants as in plants protected by invertedly repeat CMV CP RNA (Kalanditis et al. 2002). *Nicotiana benthamiana* plants were transformed with transgenes designed to produce dsRNA molecules of CMV RNA2 or CP gene sequences. Seventy five percent of the tested R_0 plants were transformed with transgenes designed to produce dsRNA molecules of CMV RNA2 or CP gene sequences. Seventy five percent of the tested R_0 plants transformed with an RNA2-derived inverted repeat construct (1534 nt CMV sequence) exhibited extreme resistance to CMV. But only a lower percentage of resistance (30%) could be obtained in R_0 lines transformed with a similar construct of shorter viral RNA2 sequence (490 nt). The CP sequences as a dsRNA construct provided resistance level reaching 50%. The

levels of resistance (100%) shown by self-pollinated (S_1) progenies obtained from most resistant R_0 plants were perfectly correlated with the expression of transgenic siRNAs. The results indicated the usefulness of inverted repeat viral transgenes as a highly efficient approach to obtain CMV resistant transgenic plants (Chen et al. 2004).

Beet necrotic yellow vein virus (BNYV, *Benyvirus*) transmitted by a fungal vector *Polymyxa betae* causes one of the devasting disease known as rhizomania of sugar beet. Of the three major groups of BNYVV isolates, the types A and B comprise four ss-genomic RNAs, while the type P has an additional fifth RNA species. The P-type is considered to be more virulent than the other two types (Heijbroek et al. 1999). In addition to conventional methods of breeding, an alternative avenue of developing transgenic plants expressing virus-derived sequences was attempted. Sugar beet plants were transformed with an inverted repeat the 0.4 kb fragment derived from the BNYVV replicase gene. The transformants displayed high levels of resistance against different strains of BNYVV when inoculated using *P. betae*. The transgenic plants exhibited high levels of resistance also under field conditions that were equal to or superior than the conventional sources of resistance, demonstrating the successful application of dsRNA-mediated resistance against BYNVV infecting sugar beet. Accumulation of siRNA was correlated with high levels of resistance to BYNVV. Further extremely low (almost undetectable) steady state levels of transgene mRNA was found in the transgenic plants, indicative of RNA-silencing mediated resistance (Lennefors et al. 2006).

The roots of transgenic *N. benthamiana* plants were found to exhibit less resistance compared to leaves against BNYVV (Andika et al. 2005). In contrast, roots of transgenic sugar beet lines were fully resistant to BNYVV and accumulated only low or nondetectable amounts of BNYVV RNA and no symptoms of rhizomania disease was seen. This may be due to the use of a part of the BNYVV replicase gene duplicated as an inverted repeat giving rise to a ds-RNA, a configuration known to function as a strong silencing inducer. The transgenic sugar beet plants showed resistance to all the three types of BNYVV isolates. The resistance provided by dsRNA was found to be better than that was provided by *CP* gene. The dsRNA-based resistance is directed to the target virus on the basis of long stretches of homologous RNA and does not need 100% identity between the transgene and viral sequence. There seems to be an advantage of using dsRNA approach, since RNA silencing does not rely on accumulation of viral proteins. This fact may facilitate the eventual release of transgenic cultivars for wider use, by eliminating the need for extensive toxicity or allergic tests (Lennefors et al. 2006).

4.1.1.4 Protection by Non-Structural Components

Along with some plant viruses, satellite viruses and satellite RNAs have also been detected in the same infected plants. The satellites cannot replicate without the help of viruses such as *Cucumber mosaic virus* (CMV) and *Tobacco ring spot virus* (TRSV) helper viruses which provide the missing information for replication. In some host plants the intensity of disease symptoms is reduced because of

the associated satellites which are considered as molecular parasites of the helper viruses. In transgenic tobacco plants expressing cDNA copies of CARNA5 (CMV-associated RNA5), inoculated with a satellite free CM, the intensity of symptoms was significantly reduced. Non-transformant plants exhibited severe mosaic and stunting symptoms. The transformed tobacco plants were resistant also to a related *Tomato aspermy virus* (TAV) (Harrison et al. 1987). Resistance was expressed when transgenic plants were inoculated with CMV via its natural aphid vector, indicating the usefulness of this approach under field conditions (Jacquemond et al., 1988; Jacquemond and Tepfer 1998); Transgenic tomato plants expressing a sat RNA of CMV showed tolerance to the virus under field conditions. The total marketable yield was increased remarkably (40 to 84%) in transgenic plants compared with CMV-infected parental lines (Stommel et al. 1998).

Transformation of plants with full-length RNA or its transcripts of the satellite virus has also been demonstrated to be effective in protecting the plants against the helper viruses. Tobacco plants expressing full-length satellite *Tobacco ring spot virus* (STRSV) or its complementary sequence as RNA transcripts, exhibited attenuated symptoms and the replication of TRSV was greatly reduced. Protection against the virus persisted for the entire life of transgenic plants. Development of transgenic plants may be a more effective disease management strategy than direct application of sat-RNAs for cross-protection of plants (Gerlach et al. 1987). The effectiveness of using combination of sequences of satellite and *CP* gene for transforming plants was investigated. The expression of *CP* and satellite sequences provided more effective protection to transgenic tobacco plants against CMV, than plants expressing the *CP* and satellite sequences separately. The risk associated with the use of satellites for viral disease control has to be considered carefully, if other strategies of disease management, though less effective, are available.

Satellite RNAs associated with plant viruses can affect the replication, pathogenesis and symptom expression in plants infected by the viruses with which they are associated. A DNA construct was introduced into 'UC82' tomato plants whose transcript contained a single full-length copy of *Cucumber mosaic virus* (CMV)-Tfn-sat-RNA, a benign variant of CMV satellite RNA. The tomato plants expressing the sat-RNA did not exhibit any symptoms of CMV, when challenged with sat RNA free strain of CMV (CMV-FL). In transgenic plants inoculated with CMV-FL strain, the symptomless phenotype was correlated to the down-regulation of CMV by Tfn-sat RNA, amplified from the transgene transcripts. The delayed resistance to CMV-77 (strain supporting a necrogenic variant of satRNA) in transgenic lines was mediated by a degradation process that targets sat RNAs in a sequence-specific manner. It appeared that there could be a correlation between a reduced accumulation level of transgenic messenger Tfn-sat RNA, the accumulation of small RNAs (approx. 23 nucleotides) with sequence homology to sat-RNAs, the progressively reduced accumulation of 77-sat-RNA in infected tissues and the transition in infected plants from diseased to healthy. The events resulting in the degradation of sat RNA sequences indicated a role for RNA silencing as the second mechanism determining resistance of transgenic tomato lines (Cillo et al. 2004).

4.1.1.5 Protection by Engineered Mild Strains of Viruses

Cross-protection is a phenomenon in which plants systemically infected with one strain of a virus are protected from infection by a second related strain of the same virus. Cross-protection, as a virus disease management, has been successfully applied in the case of *Tobacco mosaic virus* (Rast 1972), *Citrus tristeza virus* (Costa and Muller 1980), *Papaya ring spot virus* (PRSV) (Yeh et al. 1988) and *Zucchini yellow mosaic virus* (Wang et al. 1991; Lecoq et al. 1991). A mild strain HA5-1 of PRSV was artificially produced from a severe Hawaii PRSV type P strain (PRSV-P-HA) by treatment with nitrous acid. The mild strain HA5-1 protected the papaya plants effectively (90 to 100%) against severe parental strain P-HA both under greenhouse and field conditions. However, protection was strain-specific, limiting its wider application against other strains of PRSV (Yeh and Gonsalves 1984).

An attempt was made to widen the effectiveness of cross-protection also against type W strains of PRSV which can infect Cucurbitaceae, but not papaya (infected by P strain only). Chimeric mild strains were constructed from HA5-1 to carry a heterologous CP coding region and or a 3′ UTR (untranslatable region HA5-1 Wep 3U) of a type W strain that originated from Taiwan (PRSV W-CI). The chimeric mild virus HA5-1 Wep3u provided much higher level of cross protection against the type W virus W-CI than the mutant HA5-1, while still providing a high degree of cross-protection against the type P virus P-HA in cucurbits. The results indicated that broad spectrum protection against two strains [differentiated only by their ability to infect (PRSV-R) or not (PRSV-W)] can be achieved by using a chimeric mild potyvirus carrying a heterologous 3′ genomic region, since the 3′ UTR of the potyvirus is involved in replication for positive-strand viral RNA. Sequence of homology-dependent PTGS is considered an antiviral defense mechanism in virus-infected plants, playing an important role in cross-protection against the same virus or related strains (Voinnet 2001). HA5-1 Wcp 3u provided effective protection against W-CI in horn melon and squash plants than HA5-1 could provide. This may be a consequence of the effect of the identical sequences of the CP region and 3′ UTR between HA5-1 WcP3U and W-CI through a PTGS mechanism. The high degrees of protection provided by all chimeric mild strains against P-HA in horn melon and squash plants could be triggered by PTGS induced by long homologous RNA segments shared by individual chimeric mild strains and the strain P-HA. The results demonstrated that the chimeric attenuated virus strains have great potential for providing high degree of cross-protection of different strains of the same virus varying in their host range (You et al. 2005).

Interaction between plant viruses may either lead to cross-protection (interference) or synergism resulting in attenuation or intensification of disease symptoms. Members of the genus *Potyvirus* interact with a broad range of unrelated viruses, including members of the genera *Potexvirus* (*Potato virus X*), *Pararetrovirus* (*Cauliflower mosaic virus*), *Picornavirus* (*Cowpea mosaic virus*) and also other potyviruses. *Plum pox virus* (PPV) causing the sharka disease of stone fruits exists as different strains that have been classified into four main PPV groups: PPV-D (Dideron), PPV-M (Marcus), PPV-EA(EI Amar) and PPV-C (Cherry). Of

these, PPV types D and M are seen more commonly (López-Moya et al. 2000). The consequences of a PPV-M infection on plum already infected with PPV-D and vice-versa were determined by draft-inoculation of 30 Japanese plum trees with PPV-D or PPV-M isolates under quarantine conditions. The presence of one or both the isolates was monitored over a period of seven years by employing DAS-ELISA and monoclonal antibodies. The results of these tests were confirmed by RT-PCR assay with D-and M-specific primers. Real-time RT-PCR assays were performed using D-and M-specific fluorescent 3′ minor groove binder-DNA probes. The presence of PPV-D did not cross-protect Japanese plum trees against PPV-M superinfection, because this strain was detected in all trees when used as challenge inoculum. PPV-M superinfection may result in replacement of existing PPV D population or in the coexistence of the M and D-types, since PPV-M invaded the plant progressively, displacing the PPV-D population already present. The PPV-D isolate was unable to infect plants already infected by PPV-M isolate. No synergism was observed between the PPV isolate types during the 7-year investigation (Capote et al. 2006).

4.1.1.6 Protection by RNA Silencing Mechanism

Citrus tristeza virus (CTV), *Closterovirus* has a positive sense ssRNA as the genome which has 12 ORFs encoding 17 different proteins. The earlier attempts to genetically engineer citrus plants expressing specific CTV genes were not successful (Dominiguez et al. 2002; Febres et al. 2003). Three of the CTV-encoded proteins, p23, p20 and CP function as suppressors of post-transcriptional gene silencing (PTGS) activity (Karasev et al. 1995; Lu et al. 2004). The protein p23 and CP function intracellularly and intercellularly respectively, while p20 can act both ways (Lu et al. 2004). The PTGS-mediated resistance in Mexican lime against CTV was reported. The resistant plants expressed CTV encoded p23 and the resistance was considered as RNA-mediated, although siRNAs were not detected in transgenic, uninoculated plants (Fagoaga et al. 2006). In a later investigation, the resistance of *N. benthamiana* plants transformed with chimeric coding and noncoding sequences from CTV was evaluated. Several independent transgene plant lines were generated, using two constructs viz., pCTV1 and pCTV2 designed to produce self-complementary transcripts. The pCTV1 contained cDNA sequences from CTV CP, p20 and 3′ UTR (untranslated region) and pCTV2 had CP p23 and 3′ UTR sequences. Heterologous recombinant *Potato virus X* (PVX) containing either homologous or heterologous CTV sequences was employed to challenge plants. Transgenic plants (T1 generation) for each construct were resistant to recombinant PVX constructs used for challenging, if PVX contained p20 or UTR for CTV2 plants or but not if PVX had CP. Likewise, T2 generation plants also exhibited resistance. The presence of transgene-specific siRNAs in the CTV1 and CTV2 plants indicated that resistance to CTV was mediated by PTGS mechanism (Roy et al. 2006).

Sense-or anti-sense-mediated RNA silencing is able to provide only low level of disease resistance. A significant improvement in RNA silencing can be achieved by introducing inverted repeat transgenes resulting in dsRNA transcripts. By using inverted repeats, application of sense RNA-mediated silencing, has become feasible

(Chen et al. 2004). *Tomato spotted wilt virus* (TSWV, *Tospovirus*) with a wide host range including several crop species has been studied to determine the viral genes that can be employed to induce silencing in transgenic plants. Of all viral genes, *N* and NS_M gene constructs resulted in resistance, albeit at low frequencies (Prins et al. 1996). When fused to a carrier mRNA-like green fluorescence protein (GFP), sequences as short as 110-nt from TSWV *N* gene efficiently induced RNA silencing and consequently virus resistance (Jan et al. 2000).

To widen spectrum of virus resistance, a new approach involving use of *N* gene sequence fragments of the four major tomato-infecting tospoviruses, TSWV, *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and *Watermelon silver mottle virus* (WSMOV), was designed. Specific primers to amplify sequential 150-bp *N* gene segments of TSWV, GRSV, TCSV and WSMOV were designed so as to overlap where the segments of the cassette were fused. The chimeric *N* gene cassette had 600-bp consisting of successive parts of the tospoviral *N* genes of the four viruses. *N benthamiana* plants were transformed via *A. tumefaciens* and challenged with the tospoviruses individually and in combination. The symptom development was monitored for 30 days using ELISA test to exclude symptomless infection. The progeny generations of resistant plants (S2 and S3) were entirely (100%) virus-free, despite a repeated inoculation using a mixed inoculum containing all four viruses. The presence of siRNAs derived from the four segments of the transgene cassette was tested in resistant and susceptible plant lines. The siRNAs originating from the different parts of the transgene could be readily detected exclusively in virus-resistant transgenic lines. In contrast, susceptible, non transgenic control plant did not accumulate any detectable amounts of siRNAs from any part of the transgene. This finding suggests transcriptional gene silencing of the transgene in the transformed plants. This investigation demonstrates that by using RNA silencing, virus resistance frequencies of over 80% of all transformed plant lines may be achieved and the transgenic plants resistant to all the four tospoviruses can be produced providing an effective solution for the problems posed by these viruses (Bucher et al. 2006).

The RNA technology was used to engineer geminivirus resistance in tobacco plants. *Tomato golden mosaic virus* (TGMV) replication was reduced in transgenic plants expressing a *Rep* antisense RNA sequence. More than 90% of the plants of one transgenic line were symptomless. Expression of an antisense RNA sequence covering the entire TGMV *Rep*, *Trap* and RE_n genes repressed *Beet curly top virus* replication in tobacco but failed to protect plants against the less related ACMV (Bejarano and Lichtenstein 1994). *Rep* antisense-mediated resistance could be engineered against the monopartite *Tomato yellow leaf curl virus* (TYLCV) in *Nicotiana benthamiana* (Bendahmane and Gronenborn 1997) and tomato (Yang et al. 2004). Transgenic cassava plants resistant to *African cassava mosaic virus* (ACMV) disease by expressing ACMV genes of *Rep*, *Trap* and RE_n in antisense orientation were generated. By linking the viral antisense RNA to the selectable marker RNA, the transgenic plants in which the antisense RNA gene was not posttranscriptionally silenced were produced. The reduction of ACMV replication and increased resistance to ACMV infection were revealed by in vitro transient assays and infection

experiments. Thus resistance to ACMV infection of cassava can be achieved with high efficiency by expressing antisense RNA against viral mRNAs encoding non-structural proteins (Zhang et al. 2005).

The possibility of using an RNA interference construct to silence the sequence region of the *AC1* viral gene to obtain common bean (*Phaseolus vulgaris*) plants with high level of resistance to *Bean golden mosaic virus* (BGMV) was explored. Transgenic common bean lines (18) with an intron-hairpin construction to induce PTGS against the *AC1* gene, were generated. The transgenic line 5.1 exhibited high resistance, when the plants were inoculated with >300 viruliferous whiteflies per plant during the whole plant life cycle. About 93% of inoculated plants were free of symptoms of viral infection at a very early stage of plant development. Transgenic specific small-interfering RNAs were detected in both inoculated and non-inoculated transgenic plants. The presence of viral DNA in transgenic plants exposed to viruliferous whiteflies for 6 days, could be detected using a semiquantitative PCR analysis. However, the virus DNA became detectable after removal of viruliferous whiteflies that were given an additional feeding period of 6 days (Bonfim et al. 2007).

4.1.1.7 Protection by Antibody Expression

Crop plants may be engineered to express resistance to diseases caused by microbial pathogens through development of transgenic plants producing recombinant proteins, pathogen-related proteins or antisense RNAs that block pathogenesis. Antibody engineering has been developed as a novel approach to produce plants resistant pathogen by expressing recombinant antibodies (rAbs) or rAb fragments that are capable of inactivating pathogens or proteins involved in pathogenesis. This approach has been shown to be highly flexible and powerful, since the cloned antibody genes can be tailored to inactivate a pathogen, when the recombinant antibody is expressed in infected plants (Fischer et al. 2000).

With development of hybridoma technology, the usefulness of monoclonal antibodies for rapid detection, identification and differentiation of microbial pathogens and plant disease diagnosis has been demonstrated in many pathosystems. It is possible to clone antibodies and express outside animals. Cloned genes may be modified by replacing or deleting codons using site-directed mtuagenesis or the PCR assay. The feasibility of using recombinant antibodies (rAbs) for enhancing resistance to viral diseases has been indicated. Monoclonal antibodies specific to plant viruses such as *Tobacco mosaic virus* (TMV) are commercially available. These antibodies can be cloned from the hybridoma cell line for protecting susceptible cultivars against invading viruses. The phage display technology provides tools to generate monoclonal antibodies tailored to specific needs and with the desired specificity to any isolated pathogen antigen The antibody genes are modified before they are tested for expression in *Escherichia coli*, yeast, mammalian or plant cells (Voss et al. 1995). The antibody engineering and phage display (Volume 1, Chapter 2) may be exploited to isolate desired antibodies against pathogen proteins involved in pathogenesis. Thus our resources for fighting plant pathogens may be

expanded, significantly, beyond the genetic resources of plants. New plant lines expressing antibodies constitute an effective barrier preventing pathogen spread (Fischer et al. 2001).

Functional full-size rAbs are expressed in transgenic plants. Antibody expression useful for engineering resistance may be employed due to the possibility of selecting desired antigen-antibody interaction. Animal cell lines resistant to human immuno-deficiency virus have been successfully generated by applying this form of immuno-modulation (Duan et al. 1994). Plants expressing resistance to *Artichoke mottle crinkle virus* (AMCV) were generated by using single chain antibody fragments specific for ACMV. The scFV was expressed in the plant cytosol and both transgenic protoplasts and plants were resistant to ACMV (Tavladoraki et al. 1993). The transgenic tobacco plants expressing full-length antibodies cloned from a TMV-specific monoclonal antibody in the apoplast, were resistant to the virus. When a single-chain antibody fragment of this antibody (scFv24) was expressed in the cytosol, the resistance was more effective (Voss et al. 1995).

A strategy to achieve resistance based on the expression of scFV fragments against a conserved domain in plant viral RNA-dependent RNA polymerase (RdRp) a key enzyme in virus replication was formulated to develop plants resistant to *Tomato bushy stunt virus* (TBSV). The virus has a single positive sense RNA genome encoding five genes. The p92 TBSV protein was predicted to contain the highly conserved RdRp subdomains of fingers, palm and thumb. The rAbs specific for the palm domain was found to inhibit the TBSV RdRp activity in vitro and also in planta. The T1 and T2 progenies of transgenic *N. benthamiana* lines expressing selected scFVs either in cytosol or in the endoplasmic reticulum exhibited high levels of resistance against infections by TBSV and related viruses, *Turnip crinkle virus* (TCV) (*Carmovirus)* and *Red clover necrotic mosaic virus* (RCNMV) (*Dianthovirus*) (Boonrod et al. 2004).

4.1.1.8 Protection by Expression of Antiviral Protein Genes

Lodge et al. (1993) showed that exogenous application of pokeweed (*Phytolacca americana*) antiviral protein (PAP), a ribosome-inhibiting protein (RIP) present in the cell wall of pokeweed protected heterologous plants from viral infection. A cDNA clone for PAP was isolated and introduced into tobacco and potato plants by transformation with *A. tumefaciens*. Transgenic tobacco or potato plants that expressed either PAP or double mutant derivative of PAP exhibited resistance to infection by a broad spectrum of plant viruses such as *Potato virus X*, *Potato virus Y* and *Cucumber mosaic virus*. Resistance was effective against both mechanical and aphid transmission. Analysis of resistance in transgenic plants indicated that PAP conferred viral resistance by inhibiting early event in infection process (Lodge et al. 1993). Plants expressing a low level of a C-terminal deletion mutant of PAP showed resistance to PVX (Tumer et al. 1997). Another RIP from pokeweed PAP-II was found to be less toxic than PAP. The plants expressing PAP-II were resistant to TMV and PVX (Wang et al. 1988). Likewise, the protein PIP-2 from *Phytolacca insularis* showed antiviral activity against TMV (Song et al. 2000).

4.1.1.9 Protection by Genes Encoding Antiviral Compounds

The ribozymes constructed based on the nucleotide sequence of a viral gene, may cleave the target sequence efficiently in vitro. By integrating the ribozyme sequence into the plant genome, the invading virus can be inactivated. Nakamura et al. (1995) constructed hammerhead ribozymes designed to cleave the RNA sequence encoding the movement protein of *Cucumber mosaic virus* (CMV) and integrated them into tobacco plants by employing *A. tumefaciens* as the vector. The transformed tobacco plants showed resistance to CMV. In another investigation, tobacco plants were transformed with human interferon-induced dsRNA-dependent protein kinase (PKR) cDNA, using *A. tumefaciens*. Transgenic plants with an integrated PKR gene did not express the PKR protein to a detectable level and showed only a level of resistance to TMV and CMV (Nakamura et al. 1997). A ribozyme was incorporated into an antisense RNA to TMV and tested for the ability of the transgene to provide protection to the transgenic plants. There was no significant increase in the effectiveness of protection over the antisense RNA itself (de Feyter et al. 1996). In contrast, the constructs directing *Plum pox virus* (PPV) that contained a hammerhead ribozyme provided significantly higher level of protection compared with the antisense RNA construct (Liu et al. 2000).

4.2 Resistance to Fungal Diseases

The fungal and bacterial pathogens, compared to plant viruses, are more complex in structure and are capable of adopting different strategies directed towards overcoming host responses that may arrest the pathogen development. Development of crop plants resistant to fungal pathogens may involve (i) transformation for the synthesis of a novel protein which may directly restrict pathogen development; (ii) activation of expression of some endogenous gene normally induced following infection (iii) introduction of gene(s) to direct the synthesis of a novel low molecular weight antimicrobial compound(s) and (iv) introduction of gene (s) to detoxify or deactivate factors of pathogen origin required for pathogenesis.

4.2.1 Targeting Structural Components of Fungal Pathogens

Development of plants resistant to fungal pathogens depending on the successful interference with the assembly of components of cell wall and membrane and/or degrading them after their assembly by introducing the gene(s) encoding the synthesis of antifungal compounds forms the most frequently attempted approach. Introducing genes which encode potential antimicrobial PR protein with chitinase or β-1,3-glucanase activities has been found to be effective. Furthermore, the synthesis of chitinases and glucanases occurs in response to pathogen infection. Chitinase are considered to play a dual role, firstly by inhibiting fungal growth by cell wall dissolution and secondly by releasing pathogen-borne elicitors that may induce

further defense-related responses in the host plant. Synthesis of the chitinases is governed by single genes and hence, it may be comparatively easier to transfer these genes when compared to the transfer of multiple genes that control other defense-related compounds such as phytoalexins and lignins. Transgenic plants overexpressing chitinases of different origins, have been shown to exhibit enhanced levels of resistance to fungal infection and delayed symptom development when challenged with fungal pathogens (Jach et al. 1995; Lorito et al. 1998).

Chitin constitutes one of the major components of the cell walls of many fungal pathogens such as *Rhizoctonia solani* and it is hydrolyzed by chitinase. β-1,3-glucanase is known to degrade glucans present in the fungal cell walls. When both these enzymes are present together, the fungal growth is more effectively inhibited (Mauch et al. 1988). Tobacco and oilseed rape (*Brassica napus*) plants were transformed with the chitinase gene from beans (*Phaseolus vulgaris*) for constitutive high level expression of chitinase. The transformed plants exhibited higher levels of chitinase activity and significant increase in the level of resistance to *R. solani* causing seedling rot or damping-off disease. Further, lysis of hyphal tips of the pathogen was observed in the transgenic plants under the microscope (Broglie et al. 1991). *Brassica napus* var. *oleifera* plants transformed with the endochitinase gene from tomato showed a greater level of resistance to fungal pathogens *Phoma lingam, Sclerotinia sclerotiorum* and *Cylindrosporium concentricum* compared with nontransformed control plants under field conditions (Grison et al. 1996). A rice chitinase cDNA (RCC2) under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter was used to transform cucumber plants via *A. tumefaciens*. The transformants showed higher level of resistance to *Botrytis cinerea* and in some transformants spread of the disease was entirely arrested. Resistance to *B. cinerea* in cucumber due to chitinase cDNA was inherited in a Mendalian manner (Tabei et al. 1997). Likewise, rose plants expressing a rice gene encoding a basic (Class I) chitinase exhibited resistance to black spot disease caused by *Diplocarpon rosae*. The level of resistance to the disease depended on the extent of chitinase gene expression in transgenic rose lines (Merchant et al. 1998).

The efficacy of chitinase gene transformation as a strategy for improving levels of resistance of plants to diseases appears to be influenced by several factors such as plant species, type of chitin protein expressed and sensitivity of the fungal pathogens. Three chitinase genes originating from petunia (acidic), tobacco (basic) and bean (basic) were used separately to the transform three lines of cucumber cv. Endeavor. The transformed lines did not exhibit any enhancement of resistance, when challenged with *Alternaria cucumerina, Botrytis cinerea, Colletotrichum lagenarium* and *Rhizoctonia solani*. In contrast, the carrot cvs. Nanco and Golden stripe transformed with chitinase genes from bean and tobacco exhibited resistance to *B. cinerea*, *R. solani* and *Sclerotium rolfsii*. The transformants expressing the chitinase gene from petunia exhibited no increase in resistance levels to these pathogens compared to nontransformed plants. The results suggested that the basic chitinase proteins may be more effective in providing effective protection against the fungal pathogens only in some plant species (Punja and Raharjo 1996).

Grapevine plants were transformed with rice chitinase gene (*RCC2*) (Class I) by introducing *RCC2* into somatic embryos of grapevine cv. Neo Muscut by

agroinfection. Two transformants exhibited higher level of resistance to the powdery mildew pathogen *Uncinula necator*. The conidial germination, mycelial growth and conidial formation were suppressed in the transformants as revealed by electron microsope observations. Furthermore, the transgenic lines showed resistance to anthracnose disease caused by *Elsinoe ampelina*, resulting in decrease in the disease lesions (Yamamoto et al. 2000). The class I chitinase cDNA (*RCC2*) of rice driven by a CaMV 35S promoter was introduced into cucumber via *A. tumefaciens* transformation. Cucumber lines resistant (CR32), intermediate resistance (CR3) and susceptible (CR20) to gray mold disease were selected. The rice chitinase levels were higher in CR3 and CR32 showing resistance than the susceptible CR20 plants, as indicated by the results of ELISA test. Studies with in situ indirect fluorescent antibody technique and three dimensional fluorescence microscopy showed a homogenous distribution of cell expression of RCC2 to a high level in the epidermal and mesophyll cells of resistant (CR32) plants, whereas in susceptible lines the expression of RCC2 was less. The growth of *B.cinerea* within the leaf tissues of resistant lines was suppressed. The results suggested that the high level of expression and intracellular localization of the rice chitinase might result in enhancement of resistance to *B. cinerea* in transgenic cucumber plants (Kishimoto et al. 2002).

Expression of two plant defense genes may be expected to protect plants more effectively against infection by microbial pathogens. Transgenic tobacco and tomato plants expressing tobacco β-1,3-glucanase and bean chitinase genes exhibited enhanced resistance to fungal diseases (Ouyang et al. 2003). In a further study, the tobacco *AP24* osmotin gene and a bean basic chitinase gene were introduced into tomato A53 susceptible to wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* (FOL) via *Agrobacterium*-mediated transformation to generate genotypes with enhanced resistance to FOL. Molecular characterization by PCR and Southern blot hybridization confirmed the stable integration of the transgenes into the genome at various insertion sites (Fig. 4.1). Forty one of 44 putative transgenic plants showed

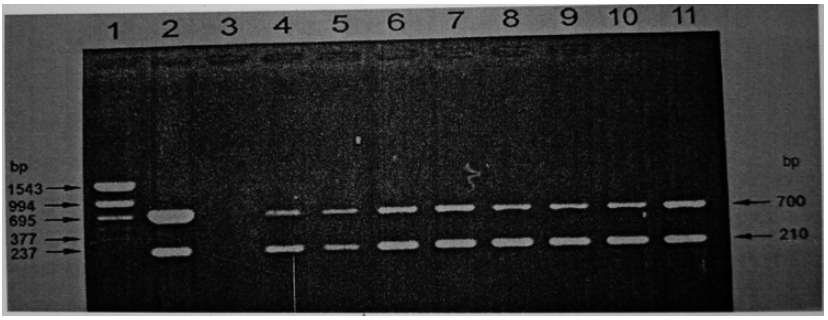

Fig. 4.1 Multiplex PCR analysis of the fragment (700-bp) from the tobacco osmotin gene (*AP24*) and the fragment (210-bp) from bean chitinase gene in transgenic tomato plants
Lane 1: Molecular weight size markers (standard); Lane 2: pHAC35 plasmid as positive control; Lane 3: Untransformed control plant; Lane 4 to 11: Eight randomly selected transgenic plants. (Courtesy of Ouyang et al. 2005; American Society of Horticultural Science, Alexandria, USA)

the presence of transgenes. Transgenic tomato plants with a single insertion of a dual transgene construct T-DNA showed significantly improved resistance against FOL. The transgenes were stably expressed in two successive generations (Ouyang et al. 2005). It is possible to increase resistance of cultivars by transformation with dual combination of PR-genes.

Different species of *Trichoderma* have been demonstrated to be effective biocontrol agents of several fungal pathogens. They constitute a potential source of antipathogenic genes. They are known to produce antibiotics and several enzymes such as chitinases, proteases, glucanases and mannanases that directly affect the growth of fungal pathogens. Transgenic tobacco and potato plants overexpressing an endochitinase (CHIT42) from *T. harzianum* were found to be highly tolerant to *Alternaria alternata, A. solani and B. cinerea* (infecting aerial plant parts) and *Rhizoctonia solani* (soilborne) (Lorito et al. 1998). Transgenic apple lines expressing the endochitinase gene (*ech42*) from *T. harzianum* were more resistant to apple scab, caused by *Venturia inaequalis*, than nontransformed Marshall McIntosh apple. The presence of the transgene was verified by applying ELISA, PCR and Southern blot analysis (Bolar et al. 2000). Two cultivars of apple, Galxy (scab-susceptible) and Ariane (carrying *Vf* resistance) were transformed with endochitinase and exochitinase genes derived from *T. atroviride*. The transgenic lines were analyzed for the expression of both genes and resistance to *V. inaequalis* races 1 and 6. A negative correlation between growth of transgenic lines and endochitinase activity was observed. All lines expressing high endochitinase activity showed a significant reduction of scab symptoms (Faize et al. 2003). Since the enhancement of resistance/tolerance of plants transformed with chitinase genes was quantitatively modest in most cases necessitating the use of gene combinations to increase the levels of resistance significantly. Transgenic tobacco plants over expressing singly or in combination, two endochitinases from *T. harzianum* viz., CHIT33 and CHIT42 were generated. Five independent F3 homozygous *pschit33* and *pschit42* lines showed significantly enhanced resistance to both a fungal pathogen *R. solani* and a bacterial pathogen *Pseudomonas syringae*. No synergistic effect in pschit33x 42 lines could be found, but the level of resistance was intermediate to their parental lines. The results suggested that the increased resistance in tobacco lines overexpressing *pschit33* or *pschit42* may be due to the systemic induction of PR proteins and increase of cell wall-associated annionic peroxidase in the transgenic *pschit* lines (Dana et al. 2006).

Some of the PR-proteins induced by exogenous application of salicylic acid (SA) which is known to function as endogenous transduction signal, are hydrolytic enzymes such as β-1,3-glucanase. These proteins have the potential to enhance disease resistance when overexpressed in transgenic plants (Ryals et al. 1996; Wobbe and Klessig 1996). The promoter of PR-2d, a β-glucanase gene of tobacco, contained in the transgenic tobacco was significantly activated by SA. The PR-2d promoter/uid A (GUS) genes construct was introduced into the cucumber genome. Activation of the PR2-d promoter in transgenic cucumber in response to inoculation with biotrophic fungal pathogens and after SA treatment was investigated. Both the endogenous and free SA levels and its conjugate salicylic acid glycoside (SAG) registered sharp increases. On the other hand, inoculation with *Erysiphe polyphage* increased GUS

activity. The results support earlier findings that endogenous SA is involved in the signaling of the plant defense responses to infection by microbial pathogens (Yin et al. 2004).

Infection of plants by fungal pathogens may be facilitated by oxalic acid through a number of routes including acidification to accelerate cell wall-degrading enzyme activity through pH-mediated tissue damage or via sequestration of Ca^{2+} ions (Dutton and Evans 1996). Oxalate oxidase belongs to the germin family of proteins that catalyze degradation of oxalic acid to produce CO_2 and H_2O_2. Oxalate oxidase was considered to have a role in the defense responses of plants to pathogen attack. Transformation of soybean, tobacco and sunflower with a wheat oxalate oxidase gene resulted in enhancement of resistance to *Sclerotinia sclerotiorum* (Zaghmout et al. 1997; Donaldson et al. 2001; Hu et al. 2003). Induction of plant defense protein was observed in transgenic sunflower plants (Hu et al. 2003). The involvement of oxalic acid in the pathogenesis of many *Sclerotinia* species has been indicated. Hence, the ability of transgenic plants expressing an oxalic acid-dergrading oxalate oxidase to provide protection against the fungal pathogens was assessed. The expression of a barley oxalate oxidase in transgenic peanut increased resistance to exogenously applied oxalic acid (upto 20 folds to pathophysiological levels). Transgenic peanut plants showed higher level of resistance to *Sclerotinia* blight disease caused by *S. minor* as determined by lesion size in detached leaf assays. Peanut transformants did not show any deleterious effects due to the expression of oxalate oxidase and hence it was used as a sensitive marker for following transformation and regeneration. A sensitive fluorescent enzyme assay was used to quantify expression levels for comparison to the colorimetric procedure (Livingstone et al. 2005) [Appendix].

4.2.2 Use of Genes for Antifungal Proteins with Different Functions

4.2.2.1 Ribosome-Inhibiting Proteins

The family *Phytolacceae* includes the genus *Phytolacca* considered as a good source of ribosome-inhibiting proteins (RIPs). They may inhibit protein synthesis by depurinating a specific residue near the 3′ end of 28S ribosomal RNA. They are present in fairly high concentrations in seeds and exhibit high levels of antifungal activity. They have selective action on foreign ribosomes (Bieri et al. 2000). The antiviral activity of RIP expression in planta has already been discussed elsewhere in this chapter. Enhanced fungal resistance has been achieved using cereal or mutated *Phytolacca* RIPs lacking nuclease activity. No cytotoxic effects associated with RIPs were observed (Nielson and Boston 2001). Tobacco plants expressing an RIP from barley seeds were tolerant to *Rhizoctonia solani* (Logemann et al. 1992).

Transgenic expression of the RIP from the pokeweed antiviral protein (PAP) was shown to be phytotoxic resulting in a stunted and mottled plant phenotype. A new gene encoding an RIP isolated from the leaves of *P. heterotopala* was expressed in tobacco under the control of the wound-inducible promoter of the bean

polygalacturonase-inhibiting protein I gene to enhance the level of resistance of fungal pathogens. The RIP isolated from *P. heterotopala* (PhRIPI) had direct antifungal activity. The PhRIPI significantly inhibited germination of *B. cinerea* conidia. The mechanism could be due to the complex interaction between the RIP and fungal cells. In the transgenic tobacco plants, the expression of PhRIPI transgene was detected and the accumulation of the protein occurred in the damaged tissues surrounding the infected areas. Consequently there was a significant reduction in the infected leaf areas. Expression of PhRIPI effectively reduced damage caused by *Alternaria alternata* and *B. cinerea*. RT-PCR and Western blot analyses revealed the expression of the RIP transgene following wounding and infection by pathogens. This result indicated that the controlled expression of the PhRIPI, due to the use of wound-inducible promoter, avoided the harmful effects of RIP resulting in apparently normal plant phenotypes. This strategy can be applied as effective procedure to counteract the pathogenic potential of wide range of pathogens as varied as viruses and even nematodes and insects (Corrado et al. 2005).

4.2.2.2 Polygalacturonase-Inhibiting Proteins (PGIPs)

Fungal pathogens produce endopolygalacturonases (endo-PGs) which have important role during early stages of pathogenesis, spreading into the host tissue in advance of the invading fungal mycelium. Polygalacturonase-inhibiting proteins (PGIPs) are basic proteins present in the cell wall of most dicotyledonous plants. PGIP is structurally similar to several resistance gene products, as it belongs to the super family of leucine-rich repeat (LRR) proteins (Mattei et al. 2001). The PGIPs accumulating at the site of infection may function as elicitor molecules by facilitating fungal PGs to increase the elicitation of plant defense responses (Cervone et al. 1987).

Tomato plants were transformed with pear fruit polygalacturonase-inhibiting proteins (pPGIP). Transgenic expression of pPGIP induced abundant accumulation of the heterologous protein in all tissues and it did not influence the expression of the endogenous tomato fruit PGIP (tPGIP). All transgenic tissues exhibited the presence of pPGIP in the cell wall protein fraction. The expressed pPGIP was active in both leaf and fruit tissues as an inhibitor of endo-PGs from *Botrytis cinerea*. The expression of pPGIP resulted in reduction of growth of *B. cinerea* on ripe tomato fruit and tissue breakdown was reduced by as much as 15% compared to nontransformed controls. Likewise, pPGIP expression in leaves reduced the lesions by about 25%. The results indicate that the expansion of lesions and tissue maceration by *B. cinerea* could be arrested by inhibiting the fungal PGS by the expression of heterologous PGIP (Powell et al. 2000).

The apple PGIP1 inhibited PGs secreted by *Verticillium dahliae* grown on potato cell walls and pectin. Hence, the possibility of employing apple PGIP1 to confer resistance against wilt disease caused by *V. dahliae* affecting potato was explored. Apple PGIP1 expressed in transgenic tobacco was able to inhibit PGs of *V. dahliae* successfully. Then, transgenic potato cv. BP1 plants containing apple *pgip1* gene under control of the constitutive enhanced CaMV35 promoter were generated. The

PCR and Southern blot analyses confirmed the stable integration of the apple *pgip1* transgene into potato gene. The extracts prepared from all except one transgenic lines successfully inhibited PGs of *V. dahliae*. Active PGIP1 was expressed both in leaves and roots of transgenic potato plants. The apoplastic localization of PGIP1 activity in the *pgip* transgenic potato plants was observed. Six transgenic potato lines showed reduced disease symptoms compared with controls and other lines grown in the sick-soil. However, the reduction in disease intensity could not be attributed to inhibition of *V. dahliae* PGs, although a high level expression of PGIP1 occurred in transgenic potato plants (Gazendam et al. 2004). In a later investigation, an apple *pgip* gene *Mdpgip1* isolated from apple cv. Granny Smith was used to transform tobacco via *A. tumefaciens* – mediated transformation. The mature MdPGIP1 protein purified from tobacco leaves inhibited PGs from *Colletotrichum lupini* infecting lupins, *Botryosphaeria obstusa* and *Diaporthe ambigua* infecting apple. MdPGIP1 was considered an attractive tool for fungal resistance through genetic modification, since it is derived from apple fruit that are already consumed raw by the people (Oelfose et al. 2006).

4.2.2.3 Small Cysteine-Rich Proteins

The small cysteine-rich proteins constitute a separate group of antifungal peptides, some of which are chitin-binding proteins, plant defensins and thionins. Thionins are cysteine-rich proteins present in several plant species. Over expression of α-thionins gene from barley in tobacco enhanced resistance in *Arabidopsis* against *Fusarium oxysporum* (Epple et al. 1997). The plant defensins are structural and functional homolog of insect and mammalian proteins which have been demonstrated to have a role in host defense. A well characterized plant defensin Rs-AFP2 was isolated from *Raphanus sativus*. Transgenic tobacco plants expressing the transgene were resistant to *Alternaria longipes* (Broekaert et al. 1995).

4.2.2.4 Nonspecific-Lipid Transfer Proteins

The nonspecific-lipid transfer proteins (NsLTPs) are included in a family of proteins with diverse functions. Some of them have the ability to transfer phospholipids between membrane vesicles and organelles, whereas others are upregulated during infection in plants (Garcia-Olmedo et al. 1995). A cysteine-rich antimicrobial protein *Ace*-AMP1 from onion (*Allium cepa*) seeds is homologous to plant Ns-LTPs. This protein has strong antimicrobial activity compared to Ns-LTPs from radish, wheat and maize seeds (Tassin et al. 1998). Further, the possibility of involvement of Ns-LTP in eliciting defense response in some host plant species as specific lipid sensors (Maldonado et al. 2002) and as competitors with elicitins for specific receptors on membrane (Buhot et al. 2001) has been reported. Scented geranium (*Pelargonium* sp.) and rose (*Rosa hybrida* cv. Carefree Beauty) transformed with the gene for *Ace*-AMP1 showed resistance to *Botrytis cinerea* and *Sphaerotheca pannosa* respectively (Bi et al. 1999; Li et al. 2003).

The rice cultivar Pusa Basmati 1 was transformed with the gene for *Ace*-AMP1 from onion, coding for an effective antimicrobial protein (AMP) homologous to Ns-LTPs. The gene for *Ace*-AMP1 was cloned under an inducible rice *PAL* (phenylalamine-ammonia lyase) or *Ub1* (a constitutive maize ubiquitin) promoter. Accumulation of *Ace*-AMP1 predominantly in the extracellular space in transgenic rice plants was demonstrated by immunolocalization using antibodies against *Ace*-AMP1. The *Ace*-AMP1 protein caused morphological deformation of the growing hyphae followed by membrane permeabilisation in the blast pathogen *Magnaporthe grisea*. The transgenic rice lines showed enhanced resistance as reflected in a reduction of 86% in disease symptoms due to *M. grisea*. Furthermore, the transgenic rice lines expressing *Ace*-AMP1 exhibited resistance to another serious fungal disease sheath rot caused by *Rhizoctonia solani* and the bacterial leaf blight induced by *Xanthomons oryzae* pv. *oyrzae* (*Xoo*) with reduced disease intensity of 67% and 82% respectively. Another desirable feature of the transgenic rice lines is the retention of all agronomic parameters of nontransformed Pusa Basmati 1 without any apparent adverse effect on the rice plant phenotype (Patkar and Chattoo et al. 2006).

4.2.2.5 Genes for Modification of Host Metabolism

The biosynthetic capacity of the host plant is enhanced by transferring genes from other plant species to effectively contain the invasion of fungal pathogens. The plants produce a wide variety of compounds related to disease resistance, following interaction with the pathogens. Phytoalexins form an important group of compounds believed to be involved in the development of resistance to diseases. Stilbenes, a class of phytoalexins known to be present in grapevines and peanut, are produced by the enzyme stilbene synthase. Tobacco plants transformed with a gene for stilbene synthase from grapevine were able to synthesize the stilbene phytoalexin resveratrol. A reduction of 60–80% in the intensity of gray mold disease caused by *Botrytis cinerea* was observed in one of the transgenic tobacco expressing stilbene synthase (Hain et al. 1993). Tomato plants (cv. Vollendung) were also transformed with two grapevine stilbene genes. These transgenes expressed in transgenic tomato plants following inoculation with *Phytophthora infestans*, wounding, induction with elicitor, or UV radiation. Inoculation with *P. infestans* and *B. cinerea* resulted in strong accumulation of stilbene synthase mRNA which could be detected in the leaves after 30 min, reaching maximum at 72 h after inoculation. Resveratrol accumulation reached maximum at 44 h after inoculation and its presence reduced the infection by *P. infestans*. Results suggest that stilbene synthase genes in transformed tomatoes might be regulated in a manner similar to that found in transformed tobacco plants (Thomzik et al. 1997).

For the control of rice blast disease, a similar approach was made to enhance the level of resistance to *Magnaporthe grisea*. The gene for stilbene synthase was transferred from grapevine into the protoplasts of the *japonica* cv. Nipponbare using the polyethylene glycol (PEG)-mediated direct gene transfer technique.The stilbene synthase gene was stably integrated in the genome of the transgenic rice plants and was inherited by the offspring as well. The transgenic rice plants exhibited greater

resistance to the rice blast disease (Stark-Lorenzen et al. 1997). Likewise, barley and wheat plants were transformed with the *Vst1* gene from grapevine, resulting in the production of resveratrol in the transformants The transformed plants showed greater degree of resistance to *B. cinerea* (Leckband and Lörz 1998).

4.2.2.6 Resistance Genes from Plant Sources

The wheat leaf rust resistance gene *Lr21* from *Aegilopsis tauschii* was first incorporated into wheat cultivar Thatcher via a synthetic wheat (Rowland and Kerber 1974). It is significant that no virulent isolates of leaf rust pathogen that could infect this cultivar with *Lr21* gene. Later the leaf rust-resistant germplasm lines WGRC2 and WGRC7 introgressed with an *Lr21* allele were developed (Huang and Gill 2001). As the introgression of the gene introduced a high degree of polymorphism in the region flanking the gene, the *Lr21* genetic locus of the lines WGRC2 and WGRC7 was investigated. A map-based cloning mapped *Lr21* to a gene-rich region at the distal end of chromosome arm IDS of bread wheat (*Triticium aestivum*). Molecular cloning of *Lr21* was confirmed by genetic transformation and by a stably inherited resistance phenotype in transgenic plants. Eight T1 progenies from resistant plant 1410 were tested with pathogen culture PRTUS6 and molecular marker KSUD14-STS. Fifteen plants were resistant to the rust pathogen and showed a 1.36 kb KSUD14-STS fragment. This fragment was absent in three susceptible plants. The genetic data revealed that the transgene for leaf rust resistance expressed stably and was inherited as a single locus. In addition, the transgeneic plant 1410 was found to have the same pathogenicity specificity as WGRC2 and WGRC7 genotypes (Huang et al. 2003).

The *Vf* locus from *Malus floribunda* 821 is considered as an important source of resistance to apple scab disease caused by *Venturia inaequalis*. A cluster of receptor-like genes at *Vf* locus from the *Vf*-containing *M*.x *domestica* cv. Florina were designated *HcrVf1, HcrVf2, HcrVf3* and *HcrVf4* (Vinatzer et al. 2001). The *M*.x *domestica* cv. Gala was transformed with *HcrVf2* under the control of the CaMV 35S promoter. Some transgenic lines containing the mutated HcrVf2 gene (due to a single base-pair mutation that occurred during cloning process) also exhibited scab resistance similar to the resistant cv. Enterpirse (Barbieri et al. 2003). Later study also indicated that constitutive expression of the *HcrVf2* gene under the control of the strong CaMV 35S promoter was capable of conferring apple scab resistance under greenhouse conditions. The *HcrVf2* gene, one of four resistance genes mapping at the *Vf* locus conferred resistance in the scab susceptible cv. Gala. Of the five transgenic lines, the Ga2-5, Ga2-8 and Ga2 showed very high levels of resistance. The line Ga2-2 was found to be as resistant as the *Vf* cultivars. The results demonstrated that direct gene transfer between cross-compatible species can be viable. This investigation was considered to represent a step toward a gene therapy (i.e. restoring resistance where lost) of the scab-susceptible apple cultivars, while maintaining the horticultural and fruit-quality traits (Belfanti et al. 2004).

The Rop proteins of plants, small GTPases (guanosine triphosphatases) have emerged as an important molecular switch in plant signal transduction. They share

high overall similarity with mammalian Rac, a subfamily of Rho GTPase (Yang 2002). The *Rop* gene has been designated *Rac* gene also in literature. Overexpression of the constitutive active form of *OsRac1* in rice led to HR at points of infection by *Magnaporthe grisea*, race 007 and consequently to disease resistance via reactive oxygen species (ROS) production. In contrast, overexpression of dominant negative forms of *OsRac1* consistently resulted in increased susceptibility to *M. grisea* (Kawasaki et al. 1999; Ono et al. 2001). The gene *OsRacB* in rice was identified based on its close genetic orthologous relationship with barley *HvRacB* gene, a known negative regulator of disease resistance. The *OsRacB* cDNA was isolated to determine its function through gene expression and transgenic approach. In the rice genome, *OsRacB* present as a single copy gene shared 98% identity with HvRacB at the amino acid level. Strong expression of mRNA of *OsRacB* was recorded in the rice leaf sheath and panicles, while its expression in young and mature leaves was weak. The transgenic rice plants, upon inoculation with *M. grisea* showed a dramatic regulation in the *OsRacB* transcripts. Overexpression of *OsRacB* resulted in enhancement of symptom severity. In addition, fluorescence microscopy of green fluorescent protein (GFP): OsRacB-transformed onion cells and *Arabidopsis* protoplasts revealed the association of OsRacB with plasma membrane. The results suggested that functioning of *OsRacB* requires its localization in plasma membrane and it functions as a potential regulator for a basal disease resistance pathway in rice (Jung et al. 2006).

Plant seeds have been reported to contain proteins with antimicrobial properties such as chitianses and β-1,3-glucanases, defensins, thionins, lipid transfer proteins and ribosome-inhibiting proteins and the genes encoding these proteins have the potential for use against plant pathogens. Motherwort (*Leonurus japonicus*) a medicinal herb used for treating some human diseases in China was tested for the presence of antimicrobial protein. A novel small protein (7.8 kDa) LJAMP1 purified from the seed was found to inhibit the growth of several fungal pathogens including *Alternaria alternata, Cercosporidium personatum* and *Aspergillus niger.* The cDNA for this protein was cloned and the LJAMP1 protein was partially similar to a series of napin-like proteins from plants. The gene *LJAMP1* was constitutively expressed in transgenic tobacco. The transgenic tobacco plants were resistant to *A. alternata* and also the bacterial pathogen *Ralstonia solanacearum*. The resistance of transgenic tobacco plants confirmed the antifungal and antibacterial effects of the protein encoded by *LJAMP1*. As there was no visible adverse effect on the growth of transgenic plants, LJAMP1 has the potential for application in plant protection through genetic engineering approach (Yang et al. 2007).

4.2.2.7 Proteins of Microorganisms

Antifungal proteins are produced not only by the plants, but also by many microorganisms which are ubiquitous in nature. The antagonistic microbes are known to produce different kinds of antimicrobial compounds and their genomes have specifically evolved to attack fungal and bacterial plant pathogens. *Aspergillus giganteus* isolated from the soil of a farm in Michigan, produces antifungal protein (AFP) which has

been well characterized (Lacadena et al. 1995). The AFP encoded by the *afp* gene has been found to have antifungal activity against several economically important fungal pathogens, including *Magnaporthe grisea* causing rice blast disease. The transgenic rice plants constitutively expressing *afp* gene were generated via *A. tumefaciens*-mediated transformation. The presence of AFP protein in the leaves of transgenic rice plants was detected by Western blot analysis, using polyclonal antibodies raised against the purified AFP protein. Constitutive expression of the *afp* gene in rice resulted in higher level of resistance to *M. grisea* in detached leaf assay. Among the transgenic lines, the line 10* harboring the synthetic *afp* gene was more resistant to rice blast pathogen. There appeared to be a correlation between *afp* gene expression level and disease resistance. The mechanisms of resistance due to the AFP protein was hypothesized to be due to inhibition of fungal growth, but not inhibition of germination of conidia of *M. grisea* at the infection site (Coca et al. 2004).

In a similar study, the activity of the promoters from three maize PR-genes *ZmPR-4*, *mpi* and *PRms* in transgenic rice expressing *afp* gene from *A. giganteus* was evaluated. Chimeric gene fusions between the maize promoters and β-glucuronidase reporter gene (*gusA*) were prepared. The *ZmPR4* promoter was found to be strongly induced in response to infection by *M. grisea*, treatment with fungal elicitors and mechanical wounding as revealed by histochemical assays of GUS activity in transgenic rice plants. The *mpi* promoter was responsive to infection and wounding, while PRms was not responsive to any of the treatments tested. The transformants expressing the *afp* gene under the control of *ZmPR4* promoter exhibited resistance to *M. grisea* to different degrees, suggesting exploitation of pathogen-inducible expression of the *afp* gene in rice plants may be effective for the protection against *M. grisea* (Moreno et al. 2004).

4.2.2.8 Recombinant Antibodies

The expression of antifungal proteins (AFPs) individually has been reported to delay the appearance of disease symptoms and the level of protection is not high. Monoclonal and recombinant antibodies against specific pathogen proteins have been produced in different expression systems, including plants (Giddings et al. 2000). Expression of single-chain (scFv) antibodies against viruses in transgenic plants resulted in effective protection against the viruses. The effect of expression of the antibodies specific to fungal cell wall proteins has been assessed in the interaction between *Arabidopsis thaliana* and *Fusarium oxysporum* f.sp. *matthiolae* (FOM). A chicken-derived scFV antibody specific to antigens displayed on the *Fusarium* cell surface was isolated from a pooled immunocompetent phage display library. The CWP2 a high affinity antibody that reacted strongly with cell wall proteins (CWPs) was identified. The selected antibody was fused to three AFPs and the resulting constructs (Chi-CWP2, AG-CWP2 and RS-CWP2) were expressed in bacteria to evaluate their antifungal activities. The mycelial growth of *FOM* was drastically arrested by each of the three fusion proteins in vitro. Disruption of fungal wall structures by the AFP-CWP2 fusion proteins was clearly visualized by mycelium fluorescence labeling providing strong evidence for the inhibitory activity. Transgenic *A. thaliana* plants expressing CWP2

or individual AFPs showed moderate resistance to *FOM*, compared to control plants expressing GFP. The resistance conferred by AFP-CWPs was specific to *FOM*, since the transgenic plants were susceptible to *Sclerotinia sclerotiorum*. The results indicate that the antibody fusion proteins may provide protection to other fungal pathogens also, if appropriate fusion proteins are employed (Peschen et al. 2004).

4.2.2.9 Antifungal Viral Genes

The effectiveness of an interstrain inhibition system known as 'killer proteins' (KPs) from *Ustilago maydis* viruses (Bruenn 2002) has been assessed to enhance resistance against smut fungi infecting wheat. Three non-homologous genes from three different viruses encoding proteins KP1, KP4 and KP6 with antifungal properties were identified. KP4 was incorporated into two swiss spring wheat varieties Golin and Greina cultivars susceptible to stinking smut disease caused by *Tilletia caries*. Specific antifungal activity of the *KP4* gene product in the transformed lines was demonstrated in vitro and in planta in the glasshouse (Clausen et al. 2000). In a later investigation, the transgenic lines expressing kp4 protein did not segregate for the transgenes and they were considered to be homozygous for *kp4* gene. The antifungal specificity of the KP system was demonstrated with both transgenic plants. KP4 in the transgenic wheat plants could inhibit the growth of *T. caries* and *Ustilago tritici* (loose smut). The transgene behaved as a quantitative resistance gene and highly specific for the fungal pathogens belonging to Ustilaginales. The antifungal effect of KP4 could be inferred by enhanced level resistance of transgenic lines under field conditions. This study appears to be the first reporting the usefulness of genes of fungal viruses in enhancing resistance of crop cultivars to fungal pathogens (Schlaich et al. 2006).

4.2.2.10 Expression of Synthetic Antimicrobial Peptides

The synthetic analogs of antimicrobial peptides offer more target specificity and increased efficacy at lower concentrations (Cary et al. 2000). Crude protein extracts from leaf tissue of transgenic tobacco plants expressing the synthetic peptide $D4E1$ gene significantly reduced the CFU from germinated conidia of *Aspergillus flavus* and *Verticllium dahliae* in vitro. In addition, enhancement of resistance to *Colletotrichum destructivum* (anthracnose) was observed (Cary et al. 2000). Transgenic cotton plants expressing synthetic antimicrobial peptide D4E1 were generated via *Agrobacterium*- mediated transformation. PCR products and Southern blots confirmed the integration of the $D4E1$ gene. The transcripts of $D4E1$ were detected using RT-PCR assay. Crude protein extracts from leaves of transgenic cotton plants inhibited colony formation from germinated spores of *Fusarium verticilliodes* and *V. dahliae* involved in seedlings disease complex and wilt disease of cotton. Transgenic cotton seeds inhibited extensive colonization and spread by *A. flavus* in cotyledons and seed coat. Furthermore, transgenic T1 seedlings showed significant reduction in disease symptoms due to *Thielaviopsis basicola* causing black root rot disease in cotton (Rajasekaran et al. 2005).

4.3 Resistance to Bacterial Diseases

When traditional breeding methods for developing cultivars resistant to bacterial plant diseases were found to be difficult, alternate strategies involving the application of biotechnological methods have been considered. Some of them have been demonstrated to have practical application for obtaining disease resistant lines.

4.3.1 Alien Genes of Plants

Of the 30*R* genes conferring resistance to the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), five genes have been characterized as encoding four types of proteins suggesting multiple mechanisms of R-genes-mediated *Xoo* resistance (Song et al. 1995; Yoshimura et al. 1998; Iyer and McCouch 2004; Sun et al. 2004; Gu et al. 2005). Wild rice *Oryzae longistaminata* is highly resistant to *Xoo*. The *Xa21* locus conferring resistance to all known races of *Xoo* in the Philippines and India was transferred from *O. longistaminata* to rice cultivar IR24. The *Xa21* gene coding for a receptor kinase-like protein was inherited as a single gene in transgenic progenies (Song et al. 1995; Wang et al. 1995). Transgenic plants expressing the *Xa21* gene were resistant to 29 of 32 isolates of *Xoo* from South-East Asian countries, India, Colombia and Nepal. The presence of a single member of a multigene family *Xa21* appeared to be sufficient to confer resistance to various of *Xoo* strains as deduced from the identical spectrum of resistance exhibited by the transgenic lines and the wild type, the donor of *Xa21* gene (Wang et al. 1996; Ronald 1997). In a further attempt to improve the level of resistance to *Xoo*, an elite indica rice cultivar IR72-was transformed with a cloned gene *Xa21*. The transformed rice lines showed a higher level of resistance to BLB disease due to pyramiding of *Xa21* with *Xa4* which was already present in IR72 rice cultivar (Tu et al. 1998).

The rice *R* gene *Xa13* was found to be fully recessive and confer resistance only in the homozygous status to the Philippines *Xoo* race 6 (strain PXO 99) which is virulent to most *R* genes (Khush and Angeles 1999). The investigation for cloning and functional analysis showed that the product of *Xa13* could play key roles in both disease resistance and pollen development, although disease resistance and sexual reproductive development are generally considered as separate biological processes regulated by different sets of genes. The gene *Xa13* was mapped to a DNA fragment of 9.2 kb, flanked by markers RP7 and ST12. Transformation of IRBB13 with DNA fragment encompassing the *Xa13* candidate including the promoter region isolated from IR64, produced 29 independent transformants. In order to characterize the function of *Xa13*, the expression of *Xa13* or *xa13* was suppressed in rice lines using an RNA interference (RNAi) strategy. The level of resistance in the cultivar Zhonghua 11 to PXO99 was significantly increased in nine of the 12 transgenic lines containing *Xa13*, as indicated by the reduced accumulation of *Xa13* transcripts. The *Xa13* and *xa13* were expressed to high levels in panicles and anthers, suggesting a possible role for them in the reproductive process. The transgenic plants with significant reduction in expression of *Xa13* or *xa13* had reduced spikelet fertility.

The reduced fertility was associated male sterility, since *Xa13*-and *xa13*-suppressed plants had smaller anthers compared with wild types and they produced mostly abortive pollen. The *Xa13*, recessive allele conferring BLB resistance, also plays a key role in both disease resistance and pollen development. This dual function of this gene provides a unique opportunity for exploring the cross-talk between disease resistance and male gamete development pathways (Chu et al. 2006).

The ethylene responsive factor (ERF) transcription factors have been demonstrated to have a rolein regulating the expression of PR genes. The expression of tomato Pti4 and Pti4 could be induced by *Pseudomonas* (Zhou et al. 1997; Wu et al. 2002). The GbERF from *Gossypium barbadense* was cloned and the transcripts accumulated rapidly reaching high concentration, following treatment of plants with exogenous ethylene and challenge by *Verticillium dahliae* (Qin et al. 2004). The function of GbERF was studied by generating tobacco plants expressing *GbERF*. Over expression of *GbERF* did not alter the phenotype of transgenic plants as well as their endogenous ethylene levels. In addition, transgenic tobacco plants exhibited greater level of resistance to *P. syringae* pv. *tabaci* (*Pst*). There was no correlation between expression level of GbERF and plant resistance to *Pst*. The expression profile revealed that the expression of ethylene inducible PR-genes such as *PR1b, PR2, PR3, PR4, Osmotin, CHN50,* ACC oxidase and ACC synthetase genes was altered. The results suggested *GbERF* might play a significant role in the response of plants to biotic stress rather than to abiotic stress. Furthermore, *GbERF* gene products may be involved in the regulation of a subset ethylene responsive genes related to the response to biotic stress due to pathogen infection (Qin et al. 2006).

4.3.2 Ectopic Expression of Bacterial Elicitor

The gene cluster *hrp* is required for both bacterial pathogenicity on host plants and HR elicitation on nonhost/resistant plants. This cluster encodes components of a type III secretion pathway and regulatory and secreted proteins. Harpins produced by Gram negative phytopathogenic bacteria are encoded by the genes hrpN, $hrpZ_{pss}$ and hrp_{pspL} respectively in *E. amylovora, P. syringae* pv. *syringae* and *P. syringae* pv. *phaseolicola*. The harpins are acidic, glycine-rich, protease sensitive and heat stable. Harpin N_{Ea} ($HrpN_{Ea}$) one of the four proteins secreted via the Hrp pathway is encoded by the gene *hrpN* located on the chromosome of *Erwinia amylovora* causing the fire blight disease of apple and other members of the family Rosaceae. The purified $HrpN_{Ea}$ when infiltrated into tobacco leaves, induces HR (Barny 1995). $HrpN_{Ea}$ is a known inducer of systemic acquired resistance (SAR) in plants such as *A. thaliana*. Harpin N_{Ea} from *E. amylovora* under the pathogen-inducible promoter *gst1*, was tested on nonhost *A. thaliana*. It decreased the infection by the fungal pathogen *Peronosopora parasitica* in *A. thaliana* (Bauer et al. 1999) and by *Phytophthora infestans* in potato (Li and Fan 1999). Transgenic pears expressing the elicitor harpin N_{Ea} under the control of the constitutive promoter CaMV 35S were examined to assess the effects of harpin. Transient expression of $hrpN_{ea}$ in pear

cells did not cause any apparent damage. Hence, 17 transgeneic clones with stable constitutive expression of *hrpN*$_{ea}$ were generated from highly susceptible cv. Passe Crassane. The susceptibility levels of the transgenic clones were found to be reduced significantly under in vitro conditions depending on the degree of expression of the transgene. This investigation has opened up a novel method of enhancing the resistance of pear to this important disease causing heavy losses (Malnoy et al. 2005).

Xanthomonas oryzae pv. *oryzae* (*Xoo*) produces harpin$_{xoo}$ encoded by the gene *hpaG*$_{xoo}$, containing 140 amino acids with a MW of 15.6 kDa. Harpin$_{xoo}$ induced hypersensitive cell death (HCD) and resistance to the pathogen as the other harpins (Wen and Wang 2001). In order to determine if both HCD and induction of resistance in hpaG$_{xoo}$ expressing tobacco (HARTOB) plants were correlated, transgenic plants were generated. The transgenic plants expressing *hpaG*$_{xoo}$ produced the harpin intracellularly. Resistance to *Alternaria alternata, Tobacco mosaic virus* and *Ralstonia solanacearum* increased in HARTOB, in proportion with the expression of *hpaG*$_{xoo}$, the gene *NPR1* that regulated several pathways of disease resistance and defense genes *GST1*, *Chia5, PR-1a* and *PR-1b* that were mediated by different signals. On the other hand, ROS intermediate burst, the expression of HCD marker genes *hsr203* and *hin1* and cell death did not occur spontaneously in HARTOB, though they were operative in untransformed and HARTOB plants treated exogenously with harpin$_{xoo}$. The results showed that the expression of harpin$_{xoo}$ in the tobacco plants conferred nonspecific resistance to bacterial, fungal and viral pathogens in the absence of HCD (Peng et al. 2004).

4.3.3 Genes Interfering with Virulence Mechanisms of Bacterial Pathogens

Some of the bacterial pathogens are known to produce toxic metabolites or toxins which function as virulence factors and are involved in the development of disease symptoms. *Pseudomonas syringae* pv. *tabaci* (*Pst*) produces the toxin, tabtoxin which is a powerful inhibitor of glutamine synthase. The severe systemic necrosis produced in susceptible tobacco plants is entirely due to the activity of tabtoxin. The pathogen remains unaffected by tabtoxin, because it produces an enzyme, acetyl transferase which is able to protect *Pst* by acetylating tabtoxin to an inactive form. The bacterial gene coding for acetyl transferase was introduced into tobacco. Symptom development was strongly inhibited by expression of the tabtoxin resistance gene even though the bacterium was able to multiply in transgenic tobacco plants (Anzai et al. 1989). In a later investigation, tobacco plants were transformed with *A. tumefaciens* strain LBA4404 carrying the plasmid pAPK21 which contains NPTII gene and *ttr* (tabtoxin resistance) gene. Field evaluation of transformants upto R7 indicated that resistance to the pathogen in the transformants was heritable (Batchvarova et al. 1998).

4.3.4 Antibacterial Proteins of Diverse Origin

Attempts have been made to introduce genes encoding antibacterial proteins of different origins into susceptible plants to protect them against diseases caused by bacterial pathogens

4.3.4.1 Lysozymes

The lysozymes are proteins with lytic activity against bacterial cell walls. But their lytic activity is at low levels. On the other hand, the mammalian or bacteriophage lysozymes cause more drastic effects on the bacterial cell walls. The lysozyme from bacteriophage T4 is highly active against both Gram-negative and Gram-positive bacteria. The lysozymes catalyze degradation of peptidoglycan, an important component of bacterial cell walls. Tobacco plants were transformed with the gene encoding the lysozyme of bacteriophage T4 fused with the signal sequence of barley α-amylase. The target enzyme was produced in the intercellular spaces. Multiplication of *Erwinia carotovora* was significantly reduced, although only very low concentration of the enzyme could be detected in the transgenic tobacco plants (During et al. 1993)

4.3.4.2 Thionins

Thionins from seeds and leaves of cereals are important sources of antibacterial activity. Thionins are considered to cause leakage from membranes. However, the exact mechanism of action of thionins on sensitive bacterial pathogens has not been understood. Tobacco plants transformed by introducing the gene encoding barley α-thionin, when inoculated with virulent and avirulent pathovars of *Pseudomonas syringae*, produced smaller lesions compared with those formed on nontransformed plants (Carmona et al. 1993). In contrast, the transgenic tomato plants expressing thionin did not show resistance to *Xanthomonas campestris* pv. *vesicatoria*, though 3% of the total leaf protein consisted of thionins. The absence of resistance was attributed to the intracellular localization of thionin in the transformed tomato plants (Stiekema et al. 1993).

4.3.4.3 Antibacterial Peptides from Insects

Humoral immunity can be induced in many insects by injecting viable nonpathogenic or inactivated phytopathogenic bacteria. A number of new proteins are formed in the hemolymph of these insects and these proteins have been shown to have antibacterial properties. Three types of protein/peptides – lysozymes, cecropins and attacins – were identified in the hemolymph of the giant silk moth *Hyalophora cecropia* (Bonman and Hultmark 1987). The cecropins belonging to the group of cytolytic pore-forming peptides have a broad-spectrum antibacterial activity against both Gram-negative and Gram-positive bacteria. These peptides are small in size with 35–37 amino acid residues, strongly basic and consist of three ma-

jor forms, A, B and C. Transgenic tobacco plants expressing cecropin B analog gene exhibited enhanced resistance to the wilt disease caused by *Burkholderia* (=*Pseudomonas*) *solanacearum* (Jaynes et al. 1993). Expression of cecropin gene in tobacco provided effective protection to another destructive bacterial disease (wild fire) caused by *Pseudomons syringae* pv. *tabaci*. Tobacco plants were transformed by introducing of a chimeric gene fusion cassette consisting of a secretory sequence from barley α-amylase ligated to a modified cecropin (MB39) coding sequence and placed under the control of promoter and terminator from potato proteinase inhibitor II (PiII) gene. Multiplication of the bacterial pathogen was markedly suppressed in the transgenic plants by more than 10-fold compared to control (Huang et al. 1997).

Attacins is another class of antimicrobial peptides detected in the insect hemolymph in response to bacterial infection (Engström et al. 1984). Attacins act on the outer membrane of Gram-negative bacteria by altering permeability and protein synthesis (Carlsson et al. 1998). Attacin genes have been identified in *H. cecropia* (*attacin E*), *Drosophila melonogaster* (*attacinA*) and *Trichopulsia ni* (*attacin A*). The acidic *attacin E* protein gene from *H. cecropia* was introduced into pear, apple and potato. The transgenic pear and apple plants showed reduced susceptibility to *Erwinia amylovora* (Reynoird et al. 1999; Ko et al. 2002). Potato plants expressing *attacin E* were less susceptible to *E. carotovora* pv. *atroseptica* (Arce et al. 1999). Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) is one of the most common and serious disease problems limiting citrus production. Transgenic *Citrus sinensis* cv. Hamlain plants expressing *attacin A* gene (*attA*) were generated via *A. tumefaciens*-mediated transformation. The cDNA clone was used to construct a binary plasmid vector (pCattA 2300) and the construct included the native signal peptide (SP) responsible for directing the introduced protein to the extracellular space, where the bacteria is expected to accumulate in vivo. The presence of the transgene in citrus was confirmed by PCR and Southern blot hybridization analysis in regenerated (12) plants. Transcription of *attA* gene could be established by Northern blot analysis in all transgenic plants. Seven transgenic lines out of eight, when inoculated with *Xac* (10^6 CFU/ml) exhibited significant reduction in susceptibility to citrus canker disease. No correlation between disease resistance and level of transcription of *attA* was observed. The results can form the basis for further research to explore the effectiveness of this approach as a disease management strategy against bacterial disease of crops (Boscariol et al. 2006).

4.3.4.4 Antibacterial Proteins from Microorganisms

Novel potent antimicrobal molecules have been isolated from microorganisms like yeast fungi. A killer toxin (KT) from *Pichia anomala* was isolated and characterized for its ability to inhibit microorganisms containing specific cell wall receptors (KT receptors) (Magliani et al. 1997). A KT-neutralizing monoclonal antibody (KT4) was employed to raise anti-idiotypic antibodies representing the internal image of the KT active domain. The decapeptide killer peptide (KP) derived from the sequence of a single-chain, anti-idotypic antibody acted as a functional inter-

nal image of a microbicidal, broad-spectrum KT. The antimicrobicidal property of the chemically synthesized KP was assayed against bacterial and fungal pathogens *P. syringae*, *E. carotovora*, *B. cinerea* and *Fusarium oxysporum*. KP was expressed in *Nicotiana benthamiana* by using a *Potato virus X* (PVX)-derived vector as a fusion to the viral coat protein, yielding chimeric virus particles (CVPs), displaying the heterologous peptide. The purified CVPs exhibited enhanced anti-microbal activity against the plant pathogens mentioned above in addition to the human pathogens such as *Staphylococcus auerus* and *Candida albicans*. The CVP particles provided strong protection to *N. benthamiana* against *P. syringae* pv. *tabaci* in vivo also. It is hypothesized that the PVX-engineered KP functionally exerts the antimicrobial activity in the intercellular space, inhibiting the early stages of growth of *P. syringae*. The results demonstrate the potential of the PVX-based display system which is a high-yield, rapid and efficient method to produce and evaluate microbial peptides in plants. This method represents a milestone for a large scale production of peptides through molecular farming (Donini et al. 2005).

Antimicrobial peptides from microbes have been shown to be effective against some phytopathogens. Due to their rapid degradation in vivo, their usefulness has been found to be limited. Indolicidin is a 13-residue peptide amide isolated from the cytoplasmic granules of bovine neutrophils. Rev4, a reverse peptide of indolicidin and Myp30, an analog of magainin (from frog) with strong antimicrobial and protease inhibitory activities in vitro were selected for investigating the effects of the genes expressing these peptides in tobacco and *A. thaliana*. All transgenic plants were able to grow and reproduce normally. In addition, transgenic plants exhibited higher levels of resistance to *P. syringae* pv. *maulicola* and *Phytophthora parasitica*. Furthermore, yield-enhancement was observed in transgenic plants under field conditions (Xing et al. 2006).

4.3.4.5 Antibacterial Proteins from Animal Kingdom

Plants may be transformed with genes not only from plants and microorganisms, but also from animals. The ability of human lysozyme protein to provide protection to transgenic plants against bacterial disease was assessed. The human lysozyme gene was assembled by stepwise ligation of chemically synthesized oligonucleotides. This synthesized gene was used to transform tobacco (*Nicotiana tabacum* SR1) via *Agrobacterium*-mediated transformation technique. The introduced gene was highly expressed under the control of CaMV 35S promoter, resulting in the accumulation of the product of the trasngene. The growth of the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (*Pst*) was strongly retarded in the transgenic tobacco plants and the size of the chlorotic halo induced by *Pst* was also reduced (Nakajima et al. 1997). Tobacco plants were transformed with a construct containing a human lactoferin cDNA, using *A. tumefaciens*-mediated DNA-transfer system. Transgenic plants expressing the foreign DNA, when inoculated with *Ralstonia solanacearum* required a longer incubation period for symptom expression. Production of lacteroferin protein in transformed plants was confirmed by ELISA tests. A significant positive

correlation between levels of lactoferin gene expression and disease resistance was observed (Zhang et al. 1998).

4.3.4.6 Resistance Through Transformation with Novel Genes Triggering HR-Like Responses

Constitutive expression of genes whose products act downstream from the putative receptors that initiate defense responses may lead to development of SAR. However, the resulting plants may display a few detrimental side effects indicating that it is possible to condition permanent SAR without seriously affecting plant growth or crop yield (Oldroyd and Staskawicz 1998). The expression of a *P. syringae avr* gene in *Arabidopsis* was linked to a glucocorticoid-inducible promoter. The transgenic plants developed HR-like lesions upon treatment with glucocorticoid inducers of the *avr* gene in *Arabidopsis* plants that also expressed the complementary *RSP2* disease resistance gene (McNellis et al. 1998). In another investigation, the expression of the *Phytophthora cryptogea* elicitor cryptogein with the pathogen-inducible hsr203J promoter, led to production of plants that expressed the cryptogein only when challenged with the pathogens that induced expressor hsr 203J promoter. These plants exhibited HR-like lesions around sites of infection of pathogens unrelated to *P. cryptogea*, as well as durable resistance to a wide range of unrelated pathogens (Keller et al. 1999). Bacterio-opsin, a protein capable of acting as a lesion mimic agent to a pathogen-inducible gene, when expressed in plants was obtained. By linking the expression of bacterio-opsin to wound-or pathogen-inducible promoters, the spontaneous lesion development and growth impairment associated with constitutive expression of bacterio-opsin were eliminated, retaining the ability for enhancement of disease resistance (Rizhsky and Mittler 2001). These investigations indicated the feasibility of utilization of inducible expression of elicitin or lesion-mimic genes for increasing the level of resistance of transgenic plants to disease (s).

In a later investigation, it was demonstrated that by linking the expression of the yeast poly (A) binding-protein gene (*PAB1*) to the SAR status of the plant it was possible to condition the plant for constitutive SAR, while avoiding the deleterious effects associated with constitutive expression of the cytotoxic gene. A feedback-regulated *PAB1* (FRP) gene was assembled and introduced into tobacco and *Arabidopsis*. The regulation entailed the linking of the expression of the *PAB1* gene to control the *lac* repressor. By linking *lac* repressor-expression to the disease resistance state of the plant, it was shown that the induction of systemic defense responses by accumulation of the yeast poly (A) binding protein would turn off the expression of the *PAB1* gene. The plants containing this system exhibited enhanced and/or constitutive expression of disease-associated genes. Increased resistance to *Erwinia carotovora* (soft rot) and *Pseudomonas syringae* pv. *tabaci* (wildfire) and *Peronospora tabacina* (blue mold) was exhibited by the transformants which displayed normal growth habit under in vitro and greenhouse conditions. This study has opened up the possibility of using cytotoxic genes whose expression may be

regulated so that resistance to diseases can be increased without any adverse effect on the plant growth and development (Addepalli et al. 2006).

Appendix: Detection of Oxalate Oxidase Activity in Transgenic Peanut Plants (Livingstone et al. 2005)

A. Colorimetric Assay

i. Transfer leaf discs (5 mm diameter) to 1.5 ml microfuge tubes; add 200 µl of assay buffer (consisting of 18 mg oxalic acid in 100 ml of 2.5 mM succinic acid, pH4.0) to each tube and incubate at 37°C for 15 min.
ii. Dispense 135 µl of developing solution containing 6 mg aminoantipyrene dissolved in 30 µl of N, N-dimethylaniline which is added to 100 ml of 0.1 M solution phosphate buffer, pH 7.0 containing 57 µl of a 140 mg per ml solution of horseradish peroxidase.
iii. Allow the reaction to continue at room temperature for 30 min.
iv. Determine the absorbance of 550 nm in a colorimeter
v. Reduce the volumes of reactants to one-half of volume required for assay using microfuge tubes and perform the assay in microtiter format, if preferred.

B. Fluorescent Enzyme Assay

Perform this assay with an Amplex Red Kit (Molecular Probes, Eugene, OR) based on the release of H_2O_2 as a measure of oxalate oxidase activity. Amplex Red forms the fluorescent compound resorufin in the presence of H_2O_2 and horseradish peroxidase.

i. Use the same assay buffer mentioned in step A(i) above, placing the leaf discs in microtiter wells and incubate.
ii. Transfer a 20 µl aliuquot of each sample with a multichannel pipettor to a fresh microtiter plate and add the reaction buffer from the kit to make up the volume to 50 µl.
iii. Dispense 50 µl of the Amplex Red/horseradish peroxidase reagent to each well and incubate for 30 min in the dark.
iv. Determine the fluorescence levels using a plate reader (Bio-Tek Instruments, Winooski, VT), using a 530 nm excitation filter and 590 nm emission filter.
v. Prepare a standard curve for calculating the enzyme activity in the samples.
vi. Measure the products of the Amplex Red reaction using a spectrophotometer at 550 nm due to the high extinction coefficient.

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Chapter 5
Induction of Resistance to Crop Diseases

Abstract Development of cultivars with genetic and transgenic resistance to combat crop diseases is beset with formidable problems. As an alternative approach to enhance the levels of resistance of cultivars with desirable agronomic attributes, induction of resistance in such cultivars has been considered as a feasible strategy of disease management. Intensive research efforts have been directed to understand the molecular mechanisms of two principal types of induced resistance viz., systemic acquired resistance (SAR) and induced systemic resistance (ISR). As in the case of genetic resistance, the natural disease resistance (NDR) mechanisms are activated by the biotic and abiotic inducers of resistance to diseases. Growth promotion by rhizobacteria in crop plants and consequent yield increases has been shown as an additional advantage, in addition to the protection of crops offered by these biotic inducers against crop diseases. Hence, the acceptability of this approach by the growers and the general public is clearly foreseen, in contrast to the genetically modified (GM) crop products. Furthermore, this approach is ecofriendly and safe, as the development of resistance in microbial pathogens to inducers is unlikely. The possibility of combining this approach with other disease management strategies has been indicated in certain pathosystems.

The usefulness and limitations of developing cultivars with genetic resistance by conventional breeding methods have already been discussed (Volume 3, Chapter 3). Although the possibility of employing genetic engineering techniques to generate disease resistant plants appears to be bright, major research efforts are confined only to a few crops such as tobacco, tomato, potato and rice. Furthermore, consumer acceptance and environmental safety requirements have placed formidable obstacles on the utility of this approach. Under these conditions, inducing resistance to disease(s) in the existing cultivars with high yield potential and quality using biotic/abiotic inducers of resistance appears to be a practical approach that can be applied to all crops. Furthermore, this approach does not involve the introduction of any foreign gene expression in susceptible plants. This approach has been demonstrated to be as safe as the use of cultivars with genetic resistance, since the same mechanisms are activated in both genetically resistant plants and susceptible plants with induced resistance.

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When the plants are treated with biotic agents such as virulent or avirulent strains of pathogens, nonpathogens, cell fragments, plant extracts and abiotic agents such as organic or synthetic chemicals, resistance both locally or systemically to subsequent pathogen attack may be induced. The chemicals may act at various points in the signaling pathways involved in disease resistance. The phenomenon of induced resistance has been differently named by various researchers earlier. Chester (1933) designated the increased resistance of infected plants to subsequent infections as 'physiological acquired immunity' which was considered as analogous to the immune response in animals. The term 'acquired immunity' was applied by Price (1936) to cross-protection between related strains of the same virus making the plant resistant to the challenging strain by the challenged strain. Ross (1961a,b) differentiated two types of resistance developed in tobacco cv Samsun NN inoculated with *Tobacco mosaic virus* (TMV, *Tobamovirus*). The resistance developed in inoculated leaves against reinfection by TMV was called 'localized acquired resistance' and the resistance induced in unioculated leaves away from the site of inoculation was designated 'systemic acquired resistance' (SAR). Later SAR was reported also in pathosystems involving fungal and bacterial pathogens. Certain families of genes collectively known as 'SAR' genes are activated and both abiotic and biotic agents may induce the same spectrum of SAR gene expression which is positively correlated to the development of a resistant state in plants. The mRNAs coded by SAR genes and encoded proteins have been characterized and the antimicrobial activities of some proteins so formed such as chitinases and β-1,3-glucanases have been established (Linthorst 1991). Thaumatin-like proteins are the products of another group of SAR genes that can disrupt membrane integrity earning the name 'permatins' (Vigers et al. 1991; Woloshuk et al. 1991). Pathogenesis-related (PR)-proteins are encoded by another group of SAR genes. The antifungal activity of PR1 – related protein from tobacco and tomato resulted in protection against the late blight pathogen *Phytophthora infestans* (Cohen et al. 1992). Expression of the pathogenesis-related-1 (*Pr1*) gene is commonly employed as marker for SAR in tobacco and *Arabidopsis.* Sequence analysis of promoters from genes coregulated with *Pr1* called the *PR1*-regulon indicated the predominance of W boxes. This W box motif TTGACC/T could be recognized by WRKY proteins which constitute a group of plant specific transcriptional regulators implicated in pathogen and stress responses, suggesting an important role of WRKY factors in regulation of the *Pr1*-regulon genes and SAR (Maleck et al. 2000). Induced resistance in its broadest sense may be defined as resistance induced by prior activation of genetically programmed plant defense pathways. The term 'priming' is applied for the defense systems that are alerted so that responses are expressed more rapidly upon challenge infections/attacks (by insects) (Conrath et al. 2002).

An inducing agent upon applications on the plant, may trigger defense mechanisms directly after challenge by the pathogen. The defense responses activated include an oxidative burst, which can result in (i) cell death, thereby trapping the pathogen in dead cells, (ii) changes in cell wall composition that can inhibit pathogen penetration and (iii) synthesis of antimicrobial compounds such as phytoalexins (Hammerschmidt 1999). Direct activation of defense-related genes may be

a resultant of induction of systemic resistance which may also lead to the priming of cells and subsequent stronger elicitation of defense responses of the host plant infected by the pathogen (Conrath et al. 2001). Induced resistance may be broadly differentiated into two forms: systemic acquired resistance (SAR) and induced systemic resistance (ISR). In response to pathogen infection or chemical application 'SAR' process is initiated, whereas colonization of plant roots by plant growth-promoting rhizobacteria (PGPR) leads to ISR. SAR is considered to be activated more commonly by pathogens causing cell death reactions, ranging from single-cell hypersensitive reaction [HR, a plant-specific type of programmed cell death (PCD)] to necrotic disease lesions. SAR is mediated by a salicylic acid (SA)-dependent process (Gaffney et al. 1993). On the other hand, ISR is mediated by jasmonate- or ethylene-sensitive pathway (Pieterse et al. 1998; Walters et al. 2005). The prospect of providing broad-spectrum disease control by activating plant's own resistance mechanisms using inducers of resistance has attracted the attention of researchers in several countries.

A better understanding of the molecular mechanisms underlying SAR has been possible using the model plant *Arabidopsis thaliana*. The ability to accumulate salicylic acid (SA) is indispensable for SAR, since *Arabidopsis* SA biosynthesis mutants *SA induction deficient 1* and *2 (sid* 1 and *sid 2)* and transgenic plants expressing the SA-degrading enzyme NahG are SAR defective (Wildermuth et al. 2001; Newrath et al. 2002). The SAR regulatory protein nonexpressor of PR genes (*NPR 1*) is activated by SA through redox changes. These changes, in turn, drives systemic expression of antimicrobial PR proteins and facilitates their secretion by up-regulating protein secretory pathway genes (Mou et al. 2003; Wang et al. 2005). The requirement of components of entirely distinct biochemical origin for realization of SAR was indicated by many investigations. SAR is specifically compromised in *Arabidopsis defective in induced resistance 1 (dir 1)* and *suppressor of fatty acid desaturase deficiency 1 (sfd 1)* exhibiting mutations in a lipid transfer protein and a dihydroxyacetone phosphate reductase respectively (Maldonado et al. 2002; Nandi et al. 2004). Further, the SAR long-disatance signaling in *Arabidopsis* appears to depend on a peptide signal system mediated by the Asp protease constitutive disease resistance 1 (CDR1) (Xia et al. 2004). The reactive oxygen species (ROS)-mediated systemic signaling net work is known to contribute to SAR. In addition, environmental conditions and plant developmental plasticity are also able to significantly influence SAR establishment (Zeier et al. 2004; Zeier 2005).

Salicylic acid (SA)-induced defense expression via nonexpressor of PR genes-1 (*NPR1*), a key mediator of SAR (Dong 2004) is present both in di- and monocotyledonous plants. *NPR1* encodes a novel protein with a bipartite nuclear localization sequence and two potential protein-protein interaction domains: an ankyrin repeat domain and a BTB/POZ (Broad-complex, Tramtrack and Bric-a-brac/POX virus and Zinkfinger) motif (Cao et al. 1997). Activity of NPR1 is dependent on the cellular redox stage. In *Arabidopsis,* AtNPR1 is present predominantly in a monomeric form that is able to translocate into the nucleus where it activates defense gene expression through interaction with transcription factors (Subramaniam et al. 2001; Zhang et al. 2003).

Exogenous application of SA triggers the translocation of NPR1 into the plant cell nucleus. After entry into the nucleus, NPR1 interacts with TGA transcription factors to mediate gene expression. The expression of a large number of genes is altered following SA treatment (Glazebrook et al. 2003; Yao et al. 2003). The PR genes are the most-studied NPR1 targets and they encode small secreted or vacuole-targeted proteins with antimicrobial properties. By using Affymetrix GeneChips (8200 genes), putative NPR1 target genes were identified by comparing transcriptional profiles of *npr1* and *npr1/35S::NPR1-GR* that were treated with SA and then the translation inhibitor cycloheximide (Chx). The induced genes were classified into groups according to their known or deduced functions. One group contains genes involved in defense, including several PR genes. Another group encloses genes encoding members of the protein secretory pathway, most of which are endoplasmic reticulum (ER)-localized proteins. These secretion-related genes include those encoding the Sec 61 translocon complex. NPR1 is also able to regulate many genes encoding ER-resident chaperones, such as BiP2 and glucose regulated protein 94 (GRP94). Induction of many genes by SA via the endogenous NPR1 was confirmed by RNA blot analysis, real-time RT-PCR (Wang et al. 2005).

Plant growth-promoting rhizobacteria (PGPR) have been demonstrated to provide effective protection to treated plants against microbial plant pathogens. One of the mechanisms of action of PGPRs in reducing disease incidence and intensity is through induction of host resistance. PGPRs applied in the soil localize at the root surface of treated plants. But they are able to induce systemic resistance (ISR) in leaves and stems far away from the root surfaces which they colonize. ISR may be defined as the active resistance dependent on the host plant's physical and/or chemical barriers activated by biotic or abiotic agents (Maurhofer et al. 1994). SAR and ISR have two phases of development. All events leading to the establishment of resistance are included in the initiation phase which is transient. During the second maintenance phase, quasi-steady-state resistance occurs as a result of events in the initial phase (Ryals et al. 1994). The resistance induced following inoculation is postulated to result from the translocation of systemic signals produced at the site of primary infection (Sticher et al. 1997). *Pseudomonas fluorescens (Pf)* induces ISR, independent of SA accumulation, but it depends on jasmonate (JA) and ethylene (ET) responsiveness (Verhagen et al. 2004). Further, ISR is also mediated by NPR1 as SAR (Spoel et al. 2003).

Various species of *Bacillus* have been reported to induce systemic resistance to diseases caused by microbial pathogens in tomato, bell pepper, muskmelon, watermelon, sugar beet, cucumber and tobacco in greenhouse or field trials. In addition to elicitation of ISR, growth promotion of treated plants has also been an additional beneficial effect of treatment with the PGPR. Elicitation of ISR by *Bacillus* spp. was associated with ultrastructural changes in plants during pathogen attack and with cytochemical alterations. Activation of some of the same pathways as in *Pseudomonas* spp. and some additional pathways has been demonstrated in different pathosystems. ISR elicited by several strains of *Bacillus* spp. was found to be independent of SA, but dependent on JA, ET and the regulatory gene *NPR1*. Nevertheless, other strains induced ISR dependent on SA and independent of JA and *NPR1*. Further, ISR

induced by *Pseudomonas* spp. does not result in accumulation of *PR1* in plants. On the other hand, ISR induced by *Bacillus* spp. in some cases, leads to accumulation of *PR1* in treated plants (Kloepper et al. 2004).

During SAR development, a massive buildup of PR proteins in vacuoles and the apoplast occurs. The basal activity of the protein secretory pathway may not be sufficient to account for the marked increase in PR protein synthesis. Hence, a coordinated up-regulation in the protein secretory machinery may be essential to facilitate proper folding, modification and transport of PR proteins. Induction of a ER-resident gene *BiP* was noted prior to the accumulation of PR, lending support to the view suggested above (Jelitto-Van Dooren et al. 1999). That an intact and responsive protein secretory pathway is required for SAR was indicated in an investigation using knockout mutants of *Arabidopsis*. The reduction in PR protein secretion was directly related to impairment of resistance against *P. syringae* pv. *maulicola* ES4326. Double mutants (*sec61αbip2* and *dad1bip2*) secreted less PR1 compared with single mutants, leading to increase in pathogen growth (Wang et al. 2005).

The endogenous levels of SA in rice, unlike most dicotyledonous plants, are high (Silverman et al. 1995). Pathogen infection does not increase the basal levels of SA, thus obscuring any potential role of SA in systemic responses in rice. Homologs of AtNPR1 have been identified in cereals. OsNH1, the closest homolog to NPR1 in rice shares 46% identity and 60% similarity with AtNPR1 and amino acids crucial for NPR1 function. Overexpression both AtNPR1 and OsNH1 conferred resistance against *Xanthomonas oryae* pv. *oryzae (Xoo)* in rice (Chern et al. 2001, 2005), suggesting the operation of a pathway in rice similar to the NPR1-mediated resistance pathway. In yeast two-hybrid systems OsNH1 interacted strongly with the transcription factor rTGA2.2. Further, rTGA factors were found to bind to the TGACG core required for SA responsiveness of PR gene promoters in rice (Fitzgerald et al. 2004). Mono- and dicot plants appear to share elements of a conserved signal transduction pathway controlling NPR1-mediated resistance. However, SA is not an effective signal for activation of defense genes and IR in cereals. Instead, a high level of endogenous SA may exert direct and/or indirect antioxidant effects to minimize oxidative damage caused by various biotic and abiotic factors (Kogel and Langen 2005).

In cereals, IR is associated with expression of various genes designated *WC1* (wheat chemical induced) genes, *BCI* (barley chemical induced) genes and *RCI* (rice chemical induced) genes. These genes are very heterogenous and few are homologous to any of the described SAR genes (Maleck et al. 2000). The resistance-inducing chemicals such as acibenzolar-S-methyl [benzol (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester, (BTH)], activate many genes which are not directly related to plant defense. The inducers of resistance chemical- and biological-prime barley and wheat for higher frequencies of a programmed host cell death response (HR) at sites of attack by biotrophic powdery mildew pathogen. HR is preceded by an H_2O_2 burst in the infected cells (Hückehoven et al. 1999). The plant protection product Oryzemate® with active ingredient probenazole (e-allyloxy-1,2-benzisothiazole-1,1-dioxide) is used to protect rice against rice blast and bacterial

blight diseases. Probenazole was found to induce SAR in *Arabidopsis* by stimulating the pathway upstream of SA accumulation (Yoshioka et al. 2001). The use of chemicals such as probenazole with a weak antimicrobial activity and capacity to induce defense responses might prove to be a preferable approach to contain the crop diseases.

5.1 Induction of Resistance to Fungal Diseases

5.1.1 Biotic Inducers

5.1.1.1 Use of Live Microbes

Induction of resistance in cucumber, muskmelon or watermelon employing *Colletotrichum lagenarium* was first demonstrated by Kuć (1990). Primary inoculation with *C. lagenarium* induced SAR to several diseases. Lignification as a mechanism of disease resistance has been demonstrated in nonhost resistance and SAR (Friend 1985). Systemic induction of SAR and formation of PR proteins has been observed in several pathosystems such as tomato-*Phytophthora infestans* (Christ and Mösinger 1989), tobacco-*Peronospora tabacina* (Ye et al. 1990), potato-*P. infestans* (Enkerli et al. 1993), tobacco-*P. tabacina* and *Phytophthora parasitica* (Alexander et al. 1993), French bean-*Colletotrichum lindemuthianum* (Dann et al. 1996) and barley- *Drechslera teres* (Reiss and Bryngelson 1996). Many of the PR proteins including PR-1, β-1,3-glucanase (PR-2), chitinases, PR-4 and osmotin (PR-5) are known to have antimicrobial activities in vitro. Constitutive expression of PR-1a in tobacco increased tolerance of transgenic plants to infection by *P. tabacina*, causing downy mildew disease and *P. parasitica*, cause of black shank disease (Alexander et al. 1993).

In green bean (*Phaseolus vulgaris*), SAR was induced by inoculation with conidia of *C. lindemuthianum* or treatment with 2,6-dichloroisonicotinic acid (INA). At 5 days after inoculation (dai) with *C. lindemuthianum*, greater increases in the activity of β-1,3-glucanases and chitinase were observed compared with INA treatment. But in the later stages (9 and 14 days) INA treatment induced still greater enzyme activities. Inoculation with *C. lindemuthianum* induced dramatic increases in the accumulation of free and glycosylated SA occurred in the first trifoliate leaves. In contrast, no change in SA content could be discerned due to treatment with INA (Dann et al. 1996). Inoculation of barley seedlings with *Blumeria (Erysiphe) graminis* f. sp. *hordei* (*Bgh)* and *Erysiphe graminis* f.sp. *tritici* (*Egt)* resulted in low levels of free SA and SA conjugates, whereas these compounds accumulated following inoculation with nonhost pathogen *Pseudomonas syringae* pv. *syringae (Pss).* However, accumulation of PR proteins occurred following inoculation with pathogens as well as with nonhost pathogen, indicating that SA accumulation in barley was pathogen-specific and independent of defense gene activation (Vallélian-Bindschedller et al. 1998). Pathogen-specific inducibility of wheat genes *PR-1.1* and *PR-1.2* by *Egt* was reported by Molina et al. (1999). These genes were induced

upon infection with either compatible or incompatible isolates of pathogen. However, there was no activation of these genes, when SAR inducers such as SA were applied.

Phytopathogens induce the synthesis of PR-proteins during interaction with several host plant species. The PR-proteins produced in corn (maize) include hydrolases (chitinases and β-1,3-glucanases) capable of degrading structural polysaccharides of the fungal cell wall (Huynh et al. 1992), ribosome-inhibiting proteins (RIPs) and zeamatin (Roberts and Selitrennikoff 1990; Guo et al. 1997). β-1,3-glucanase present in corn seed was proposed as a mechanism for inhibiting the growth of *Aspergillus flavus* (Lozovaya et al. 1998). The expression of β-1,3-glucanase and the isoform patterns in peanut seed inoculated with *A. flavus* was investigated. In the resistant genotypes (GT-YY9 and GT-YY20) significant increase in the activities of β1,3-glucanase was noted after inoculation of seeds with *A. flavus*, over that of susceptible genotypes (Georgia Green and A 100). There were more protein bands corresponding to β-1,3-glucanase isoforms in the infected seeds of resistant genotype than in infected seed of susceptible genotype. The results indicated for the first time, the association of β-1,3-glucanase activities with resistance to *A. flavus* colonization in peanut seed (Liang et al. 2005)

Accumulation of phytoalexins has been observed in resistant plants following infection by microbial pathogens, indicating their involvement in disease resistance. Coordinated expression of functionally independent genes is required, since the biosynthesis of phytoalexins is governed by several genes. Exploitation of this strategy for enhancement of disease resistance depends on the integration of multiple mechanisms and factors such as signals, signal transduction, elicitors, suppressors and detoxification (Kuć 1995). The feasibility of this approach has been shown in certain pathosystems. Inoculation of freshly harvested avocado fruits with a mutant strain of path-1 of *Colletotrichum magna* induced resistance to *C. gloeosporioides* (*Gloemerella cingulata*). The strain path-1 induced high levels of epicatechin which persisted in ripening fruits. The enhanced level of resistance was considered to be due to the inhibition of oxidation of the antifungal diene present in the fruit by epicatechin (Prusky et al. 1994). The antifungal compounds encoded by genes of mycoparasitic fungi may provide protection to plants against fungal pathogens. Chitinases produced by the biocontrol agents *Trichoderma harzianum* exhibited antifungal activities against *Colletotrichum falcatum* causing the red rot disease of sugarcane (Viswanathan et al. 2006).

Trichoderma hamatum strain 382 induced resistance in cucumber against root rot, crown rot, leaf and stem blight caused by *Phytophthora capsici*. *T. hamatum* remained spatially separated from the pathogen in the split root and blight assays, suggesting that resistance induced was systemic in nature. The effectiveness of protection provided by *T. hamatum* was equal to that offered by the chemical inducer benzothiadiazole (BTH) (Khan et al. 2004). Treatment of cotton seeds with *T. virens* effectively protected cotton plants against root rot disease caused by *Rhizoctonia solani*. Terpenoid synthesis and peroxidase activity in roots of treated plants were examined but not in the untreated control plants. The terpenoid pathway intermediates deoxyhemigossypol (dHG) and hemigossypol (HG) strongly inhibited the

development of *R. solani*, indicating that terpenoid production was the major contributor for the control of root rot disease. Furthermore, a strong correlation between the biocontrol and induction of terpenoid was revealed, when strains of *T. virens, T. koningii* and *T. harzianum* were compared. The results indicated that induction of resistance by *T. virens* occurred through the activities of terpenoids acting as elicitors (Howell et al. 2000). An 18- kDa protein isolated from the culture filtrate of *T. virens*, elicited defense responses in cotton seedling roots in either a denatured or renatured form. The amino-terminal end of this protein had highest similarity (63% identity) to a serine proteinase from *Fusarium sporotrichoides* (Hanson and Howell 2004) (Table 5.1).

Some species of *Trichoderma* are able to colonize the intercellular species of plant roots up to the cortex layer and induce systemic resistance as in cucumber (Yedidia et al. 1999). An understanding of the signaling events leading to ISR may enable genetic manipulation of *Trichoderma* strains for improving their biocontrol potential. The *tmkA* gene whose product is a MAPK (mitogen-activated protein kinase) homolog belonging to the YERK1 (yeast and fungal extracellular signal related kinase 1 loss of which resulting in pathogenicity) was isolated from *T. virens* and the loss-of-function mutants were constructed (Mukherjee et al. 2003). The role of MAPK, TmkA inducing systemic resistance in cucumber against *P. syringae pv. lachrymans (Psl)* was examined by using *tmkA* loss-of-function mutants. The mutants, though were able to colonize the cucumber plant roots as effectively as the wild-type strain, could not induce full systemic resistance against *Psl.* Interaction with plant roots increased the accumulation of *tmkA* transcript in *T. virens* and its homologs in *T. asperellum*. The results indicate that TmkA MAPK was not involved in the limited root colonization by *T. virens* which, however, required MAPK signaling to induce full systemic resistance in plants (Viterbo et al. 2005).

Trichoderma asperelllum (= *T. harzianum* 203) penetrates the roots of cucumber seedling and then colonizes the epidermis and root cortex, resulting in induction of systemic resistance (ISR) (Yedidia et al. 1999; Shoresh et al. 2005). *T. asperellum*

Table 5.1 Effect of cotton seedling root treatment with protein bands fractionated using polyacrylamide gel electrophoresis (PAGE) on induction of phytoalexins

Protein band (kDA)	Renatured*		Denatured*	
	Desoxyhemigossypol (dHG)	Hemigossypol (HG)	dHG	HG
Buffer	4.7** d	5.0 e	3.8 bc	2.8 b
6.5	10.8 ab	22.9 ab	4.1 b	2.6 bc
18.0	12.6 a	25.6 a	7.4 a	4.5 a
20.0	7.2 cd	13.9 cd	4.1 b	2.1 bc
26.0	12.2 a	24.6 a	4.5 a	4.0 ab
32.0	5.7 d	7.1 de	2.9 c	1.8 bc
42.0	10.0 ab	16.0	2.9 c	1.4 c

* Bands were either used directly (denatured) or incubated in renaturation buffer before use.
** Values are micrograms of dHG or HG per gram of root tissue; numbers followed by the same alphabet are not statistically different at $\alpha = 0.05$ as per Fisher's least significant difference.
(Source: Hanson and Howell 2004)

modulated the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR (Shoresh et al. 2005). A *Trichoderma*-induced MAPK (TIPK) gene was isolated and characterized. Sequence analysis demonstrated that the *TIPK* was homologous to *MPK3a, WIPK* and *MPK3* genes that were upregulated by pathogenic bacterial and fungal elicitors (Mayrose et al. 2004). Preinoculation of roots with *Trichoderm*a activates defense mechanisms leading to resistance to leaf pathogen *P. syringae* pv. *lachrymans (Psl).* A unique attenuated virus vector *Zucchini yellow mosaic virus* (ZYMV-AGII) was employed to overexpress TIPK protein and antisense (AS) RNA. Plants overexpressing TIPK exhibited enhanced resistance to *Psl* infection compared with control plants, even in the absence of preinoculation with *Trichoderma*. An increase in susceptibility of plants expressing *TIPK-AS* was recorded. Nevertheless, preinoculation with *Trichoderma* did not protect the AS-cucumber plants against subsequent infection by *Psl.* Southern analysis of digested DNA by several restriction enzymes showed that *TIPK* was a single-copy gene. The *TIPK* expression was silenced in AGII-AS plants inoculated with *Trichoderma*. *Psl* multiplication in *TIPK*-silenced plants preinoculated with *Trichoderma* was higher than in AGII-GFP control plants. In contrast, AGII-GFP control plants preinoculated with *Trichoderma*, had lower *Psl* population. This investigation showed that TIPK was a crucial component in the pathway of signals being transferred from the interaction site of *Trichoderma* with the plant and the protective effect of *Trichoderma* was through activation of the *TIPK* gene (Shoresh et al. 2006).

Leptosphaeria maculans (A group) causes the Phoma stem canker at the stem base of winter oilseed rape (*Brassica napa*), whereas *L. biglobosa* (B group) induces less severe lesions on upper stems (West et al. 2002). The ability of *L. biglobosa* to induce resistance in *B. napa* to *L. maculans* was assessed. Pretreatment of *B. napa* (cv. Madrigal) with spores of *L. biglobosa* delayed the appearance of lesions induced by *L. maculans* both in pretreated and untreated leaves far away. Further, the severity of disease was also reduced by the pretreatment. The results suggested that the pretreatment with the biological defense inducer can reduce local and systemic resistance to *L. maculans* with both short-term effects on the development of Phoma leaf spotting and long-term effects on the development of stem canker eight months later. The effects of the biological inducer were equal to that of the chemical inducer acibenzolar-S-methyl (ASM) in inducing resistance to Phoma stem canker in winter oilseed rape plants (Liu et al. 2006).

Antagonistic yeasts are capable of inducing resistance as evidenced by enhanced production of defense-related enzymes. *Aureobasidium pullulans* reduced the decay in apples due to *Botrytis cinerea* and *Penicillium expansum* causing gray and blue mold diseases respectively. The enhanced resistance of treated apples was associated with the transient increase in β-1,3-glucanase, chitinase and peroxidase activities commencing from 24 h after treatment (Ippolito et al. 2000). Likewise, enhancement of natural disease resistance (NDR) in strawberry to *B. cinerea* following treatment with *A. pullulans* was observed (Adikaram et al. 2002). The yeast species *Candida oleophila* (included in the commercial product Aspire) induced systemic resistance in grapefruit to *Penicillium digitatum* causing green mold disease (Droby et al. 2002). The onset of systemic resistance in fresh apples to gray mold disease

was found to coincide with increase in the activities of chitinase and β-1,3-glucanase in systemically protected tissues (El Ghaouth et al. 2003). Cytochemical investigations on the changes in the exocarp tissues of citrus fruits treated with *Verticillium lecanii* indicated the accumulation of callose and lignin-like compounds at sites of colonization by *P. digitatum,* resulting in restriction of decay development in the treated fruits compared with the untreated control fruits. *V. lecanii* and chitosan, a natural compound from crabshell showed similar ability to induce transcriptional activation of defense genes leading to the accumulation of structural and biochemical compounds at strategic sites of pathogen interaction (Benhamou 2004).

Salicylic acid (SA), a siderophore produced by *Pseudomonas aeruginosa* 7NSK2 was found to be primarily responsible for the induction of ISR to *Botrytis cinerea* in bean and this resistance was iron-regulated (Meyer and Höfte 1997). The PGPR may induce a set of defense reactions, culminating in the production of physical barriers and creation of fungitoxic environment that may adversely affect the development of microbial pathogens. Bacterization of pea roots with *P. fluorescens* strain 63-28 resulted in the wrinkled appearance and collapse of hyphae of *P. ultimum*, as visualized in electron-micrographs. Significant modifications of epidermal and cortical cell walls and deposition of newly formed barriers were observed in roots challenged with the wilt pathogen *F. oxysporum* f. sp. *pisi.* In addition to the direct antifungal activity of *P. fluorescens*, the indirect action by reinforcement of host cells and acceleration of synthesis of phenolic compounds could have contributed to the restriction of pathogen development in this pathosystem (Benhamou et al. 1996).

Sett treatment with *P. fluorescens* and *P. putida* induced accumulation of chitinase in germinating sugarcane settling. On the other hand, application of these PGPR strains induced chitinase activity systemically in sugarcane stalk tissues. The enhanced chitinase activity was related to the suppression of development of red rot disease caused by *Colletotrichum falcatum* (Viswanathan and Samiyappan 2001). Synthesis of defense proteins and enzymes were induced following treatment of tomato plant with *P. fluorescens* and challenge with *F. oxysporum* f.sp. *lycopersici* (Ramamoorthy et al. 2002). Root inoculation of *A. thaliana* with *P. fluorescens* CHA0 resulted in partial protection of leaves against *Pernonospora parasitica* causing downy mildew disease. Induction of ISR to *P. parasitica* required the synthesis of 2,4-diacetylphloroglucinol (DAPG) in *P. fluorescens,* as application of DAPG at 10 to 100 μM mimicked the ISR effect, indicating the possibility of similar mechanism operating in other pathosystems also (Iavicoli et al. 2003). The studies using several strains of *Pseudomonas* spp. showed that elicitation of ISR is typically dependent on SA and did not result in activation of *PR-1a* gene that encodes PR-1a (Van Loon and Glick 2004).

In tobacco-blue mold (*Pernospora tabacina*) pathosystem, treatment with *Bacillus pumilus* strains SE34 significantly increased levels of SA at one day after challenge inoculation with the fungal pathogen (Zhang et al. 2000). In the roots of pea bacterized with *B. pumilus* strain SE 34, challenged with *F. oxysporum* f.sp. *pisi (Fop)*, colonization of *Fop* was restricted to epidermis and outer cortex of roots, whereas in nonbacterized roots the pathogen colonized extensively the cortex, endodermis and paratracheal parenchyma cells and radiated toward the vascular stele.

This restriction of pathogen in treated plants was due to strengthening of the epidermal and cortical cell walls. In addition, cell wall appositions with large amounts of callose and infiltrated with phenolic compounds were also present in root tissues of bacterized pea plants (Benhamou et al. 1996). This bacterial strain induced systemic resistance in tomato plants against *F. oxysporum* f.sp. *lycopersici* resulting in reduction in severity of typical symptoms and number of brown lesions formed on lateral roots of treated tomato plants. By using gold-complexed β-1,3-glucanase assay, higher amounts of β1,3-glucans in the root tissue were detected , when plants were treated with strain SE34 and chitosan, compared with individual treatements and untreated control plants (Benhamou et al. 1998).

Many strains of *Bacillus subtilis* have been reported to provide effective protection against soil-borne pathogens such as *Rhizoctonia solani* and *Pythium* spp. (Asaka and Shoda 1996; Harris and Adkins 1999). Antibiotic production was considered to be primarily responsible for disease suppression by *B. subtilis.* In a later study, the nonpathogenic isolate *B. subtilis* M4 was shown to stimulate a systemic response in cucumber and tomato leading to protection against *Colletotrichum lagenarium* (anthracnose) in cucumber and *Pythium aphanidermatum* (damping-off) in tomato. The pathogens and M4 strain were not in contact with each other, thus excluding the possibility of antibiotic having a role in disease control. Treatment of plant roots with M4 strain was found to induce systemic molecular modifications. The RNA expression profiles in control and treated plant leaves were compared using the cDNA-AFLP technique. Several AFLP fragments corresponded to genes not expressed in control plants treated with M4 strain. The differential accumulation of mRNA indicated the plant reaction following perception of M4 strain. This report appears to be the first evidence for the specific modulation of gene expression by *B. subtilis* strain in tomato and cucumber triggering plant defense machinery (Ongena et al. 2005).

Inoculation of tomato plants with *Tobacco necrosis virus* (TNV) induced resistance to the late blight disease due to *P. infestans*. Accumulation of 6 PR-proteins were detected by using specific antisera and the basic fractions of these PR-proteins were inhibitory to *P. infestans*. In addition, marked increase in the activity of peroxidase (PO) both in the inoculated and uninoculated resistant upper leaf tissue was observed prior to development of SAR. Synthesis of three new PO and one β-1,3-glucanase isozyme was induced in tomato plants showing SAR (Anfoka and Buchenauer 1997). The resistance induced by TMV in tobacco against *Pernospora tabacina* appeared to depend on volatile or diffusible compound(s) (Xie and Kuć 1997). Induction of resistance by viruses in other crops such as cucumber by TNV against powdery mildew disease (Sticher et al. 1997) has been reported. Infection by TMV induced resistance in tobacco cv. Havana against powdery mildew disease. In TMV-infected plants, the cell wall hydroxyproline-rich glycoprotein (HRGP) may be associated with SAR activation against powdery mildew pathogen (Raggi 1998). A bacterially produced rice thaumatin-like protein was found to be inhibitory to several plant pathogens such as *F. oxysporum* f.sp. *cubense, Botrytis cinerea, Drechslera oryzae* and *Rhizoctonia solani* causing economically important diseases (Jayaraj et al. 2004). However, the effectiveness of this protein in protecting the plants against pathogen(s) causing crop diseases is yet to be demonstrated.

5.1.1.2 Use of Resistance Inducers from Microbes

Nonpathogen bacterium *Pseudomonas putida* BTP1, applied on the roots of bean (*Phaseolus vulgaris*) reduced the disease symptoms caused by *Botrytis cinerea* on leaves. The molecular determinant of *P. putida* involved in ISR was isolated from cell-free culture fluid after growth. The N-alkylated benzylamine derivative (NABD) purified from culture fluid mimicked the protective effect as noted with crude samples. The mutants impaired in NABD biosynthesis lost the elicitor activity, indicating the requirement of NABD for ISR stimulation (Ongena et al. 2004). The biocontrol agent *Paenibacillus alvei* K165 protects *Arabidopsis thaliana* against *Verticillium dahliae* through ISR. The components of the pathway from isochorismate and a functional NPR1 protein appeared to play a crucial role in the K165-mediated ISR. In addition, the concomitant activation and increased transient accumulation of the PR-1, PR-2 and PR-5 genes occurred following the treatment that included both the inducing bacterial strain and *V. dahliae* (Tjamos et al. 2004).

A cascade of resistance factors is induced when the host plant recognizes the presence of a potential pathogen. The compounds of pathogen or plant origin capable of triggering resistance responses are designated 'elicitors'. Cultured plant cells, fungal cell wall fragments and secreted proteins that may elicit defense gene expression have been examined and formation of defense-related gene products during plant-pathogen interaction has been detected (Volume 3, Chapter 3). Many fungal elicitors produced in culture media have been characterized. Further, the presence of elicitors has been detected in intercellular washing fluids (IWFs) from infected plants (Takenaka et al. 2003). The cell wall proteins (CWPs) of *F. oxysporum* f.sp. *ciceri* and *Macrophomina phaseolina* causing wilt and charcoal rot diseases of chickpea respectively were used to treat chickpea seedlings. Synthesis of phenol, PR-protein and activities of phenylalanine-ammonia lyase (PAL) and peroxidase (PO) were enhanced due to treatment with CWPs. Infection by both pathogens was also reduced. As the CWPs did not show any antifungal activity on the pathogens, the CWPs were considered to act as elicitors of disease resistance (Saikia et al. 2006).

The oomycete pathogens *Phytophthora* spp. and *Pythium* spp. secrete elicitins, low molecular weight proteins that act as biotic elicitors of plant defense. The elicitins (10-kDa proteins) produced by most *Phytophthora* spp. show structural similarity and induce HR in specific plant species. *P. infestans* causing late blight disease of potato, secreted an elicitor, INF1. The expression of the *inf1* gene was down-regulated in planta during the biotrophic stage. It appeared that resistance to *P. infestans* may not be mediated by a defense elicited by INF1 in the genus *Solanum* (Kamoun et al. 1997). When the tuber disks of Irish Cobler potato were treated with a fungal elicitor composed of hyphal cell-wall components from *P. infestans*, synthesis of a 51-kDa protein kinase (p51-PK) occurred. Furthermore, SA and arachidonic acid were also able to activate a protein kinase with similar migration as p51-K on SDS-PAGE, indicating the possible involvement of p51-PK in the induction of defense responses of potato (Katou et al. 1999).

The tobacco plants pretreated with cryptogein, the elicitin secreted by *Phytophthora cryptogea* were resistant to subsequent inoculation with *P. parasitica* causing black shank disease. The acquired resistance was exhibited systemically in all plant

parts (Ricci et al. 1989; Bonnet et al. 1986). Modifications of ultrastructures induced by cryptogein on suspension-cultured tobacco cells were visualized. Pectic fibrillar expansions forming a pectate gel along the primary wall of elicited cells were seen (Kieffer et al. 2000). In a later study, cryptogein-treated plants showed (i) formation of a calcium pectate gel in intercellular spaces of parenchyma, (ii) impregnation of pectin by phenolic compounds in intercellular spaces of phloem bundles and (iii) accumulation of phloem protein (P proteins) in the lumen of leaf sieve elements. These cytological changes led to formation of physical barriers effectively restricting the host tissue colonization by *P. parasitica*, when inoculated on tobacco plants treated with cryptogein. Wall appositions were also seen at most sites of attempted penetration by pathogen hyphae. In addition, severe morphological damages on the growing hyphae could be noted (Lherminier et al. 2003).

Another elicitin-like molecule is oligandrin secreted by *Pythium oligandrum*. Although oligandrin has biochemical similarities with other elicitins, it does not induce a visible HR in leaves of treated tomato plants. However, it is also translocated through vascular elements like cryptogein (Picard et al. 2000). Oligandrin-treated tomato plants became resistant to *P. parasitica* and *F. oxysporum*, because of systemically induced resistance. However, in tobacco the effectiveness of resistance induced against *Phytophthora* was less compared with that provided by cryptogein. Cytological investigations revealed both morphological alterations of pathogen hyphae and host defense reactions resulting in reduction of viability of pathogen and consequent development of resistance to the disease (Picard et al. 2000; Benhamou et al. 2001). The comparative study on the effect of oligandrin treatment showed oligandrin induced similar ultrastructural changes in tobacco plants as cryptogein resulting in induction of disease resistance (Lherminier et al. 2003).

Two types of cell wall protein (CWP) fractions from *P. oligandrum* with elicitin activity were isolated by Takenaka and Kawasaki (1994) and Takenaka et al. (2003). In order to understand the mechanism of CWP-induced resistance to diseases, global gene expression in CWP-treated roots of tomato was analyzed using an 11,520 nonredundant cDNA array. Of the 144 up-regulated genes, nine genes exhibited about eight-fold increased expression. The analysis of the up-regulated genes in CWP-treated roots showed that the expression of beta-cyanoalanine synthase (*LeCAS*) gene encoding hydrogen cyanide (HCN) detoxification enzyme was highly induced in tomato roots, not only by CWP treatment, but also by probenazole (PBZ), BTH and validamycin A (VMA). The expression of *LeCAS* was also induced after exogenous application of 1-amino-cyclopropane-1-carboxylate as the precursor of ethylene, but not either SA or MeJ. The induction of *LeCAS* expression following treatment with CWP or activators may be due to detoxification of HCN during ET production. Up-regulation of *LeCAS* gene has the potential for use as an excellent molecular marker for screening possible novel plant activators for protecting crops against microbial pathogens (Takahashi et al. 2006).

Pythium oligandrum is known to produce oligandrin capable of inhibiting the development of *Botrytis cinerea* causing grey mold disease in grapevine. This biocontrol agent (BCA) or the purified oligandrin was applied on roots and the changes induced in the ultrastructure and at molecular level were monitored. Following inoculation of grapevine leaves with *B. cinerea,* invasion of leaves was restricted

significantly providing 75% protection to treated plants. Modifications of cuticle thickness, accumulation of phenolic compounds and cell wall appositions were observed in treated plants challenged with the pathogen. No visible necrotic lesions representing HR due to elicitation treatment were noted. Expression of defense-related genes (LTP-1, β-1,3-glucanase and stilbene synthase) monitored by analyzing RNA isolated from *B. cinerea*-infected leaves of grapevine challenged or not with *P. oligandrum* or oligandrin by real-time RT-PCR assay. Expression of all three defense-related genes was enhanced to different levels by the BCA and elicitin treatments. The absence of HR following treatment with oligandrin appears to represent distinctly different plant reactivity to elicitins which have been reported to be associated with HR on plants (Mohamed et al. 2007).

By using the RT-PCR procedure, a partial cDNA of 246 bp was obtained for cloning the endochitinase gene from *T. harzianum* T5 (Viswanathan et al. 2006). This investigation indicated the possibility of using the chitinase gene of *T. harzianum* to protect sugarcane plants against red rot disease. The molecular mechanism involved in bioprotection offered by *Trichoderma* spp. to plants remains largely unclear. An elicitor secreted by *T. virens* was identified as a small protein designated Sm1 (small protein 1) which lacked toxic activity to both plants and microbes. The native purified Sm1 triggered production of reactive oxygen species (ROS) in monocot (rice) and dicot (cotton) seedlings. Expression of defense-related genes was induced by Sm1 both locally and systemically. The gene *SM1* was expressed in vitro in the fungus as well as in the plant system. Treatment of cotton cotyledons with Sm1 induced resistance in cotton to *Colletotrichum* sp. (Djonović et al. 2005).

Harpin a protein secreted by the bacterial pathogen *Erwinia amylovora (Ea)* causing fire blight disease of apple, is an acidic heat-stable, glycine-rich, 44-kDa protein encoded by the gene *hrpN*. Harpin induced SAR in tobacco and *Arabidopsis* (Dong et al. 1999). Application of harpin on harvested Red Delicious apples induced resistance to the blue mold disease caused by *Penicillium expansum* (de Capdeville et al. 2002). In a further study, the effectiveness of resistance induced by harpin in harvested apples was assessed for practical use as a method of control of blue mold disease. Harpin was applied as pre- and post-harvest spray. Fewer fruits treated with harpin were infected. Spraying harpin a few days before harvest was recommended as a promising strategy for reducing blue mold decay in storage. Harpin treatment resulted in accumulation of tannin in the epidermal and hypodermal cells and also some structural changes in the cell wall such as production of wall-like appositions (de Capdeville et al. 2003).

5.1.2 Abiotic Inducers

5.1.2.1 Plant Products

The nonhost plants that are immune to plant pathogen(s) capable of inflicting serious disease(s) in susceptible plant species may contain compounds that may be

responsible for the immunity. Milsana, a commercial preparation made from the leaves of the giant knotweed plant (*Reynoutria sachalinenesis*), is an eliciting mixture containing proteins, glycoproteins, peptides, carbohydrates and lipids that recognize and signal the presence of the pathogen, subsequently triggering defense responses in a non-cultivar-specific manner (Daayf et al. 1997, 2000). Resistance to *Podosphaera xanthii* (= *Sphaerotheca fuliginea*) causing powdery mildew disease, was induced in cucumber by application of Milsana. This resistance was correlated with increased mRNA accumulation and greater extractable enzymatic activity for chalcone synthase (CHS) and chalcone isomerase, two key enzymes of the flvaonoid pathway (Fofana et al. 2002). In a later study, accumulation of rapidly synthesized C-glycosyl flavonoid phytoalexins (cucumerins) was observed at the sites of the pathogen penetration within Milsana-elicited cucumber plants (McNally et al. 2004). The role of flavonoid phytoalexin production, liginification and chitinases in powdery mildew disease resistance in cucumber was evaluated. After elicitation of resistance by Milsana, the cucumber plants were infiltrated with inhibitors of cinnamate 4-hydroxylase, 4-coumarate:CoA ligase (4CL) and CHS. The elicited cucumber plants exhibited a high level of induced resistance. In contrast, down-regulation of CHS, a key enzyme of flavonoid pathway resulted in nearly complete suppression of induced resistance and the development of healthy haustoria within these plants was revealed by electron microscopy. Inhibition of 4CL had little effect on induced resistance. The results suggested that induced resistance in cucumber was largely correlated with de novo biosynthesis of flavonoid phytoalexin compounds rapidly (Fofana et al. 2005).

The blue green algae *Laminaria digitata* is the source of a laminarin, β-1,3-glucan laminarin which elicits defense responses in grapevine cells and plants against *Botrytis cinerea* and *Plasmopara viticola*. The development of these pathogens in infected grapevine plants was effectively arrested by laminarin. The defense responses included calcium influx, alkalinization of the extracellular medium, an oxidative burst, activation of two mitogen-activated protein kinases, expression of ten defense-related genes with different kinetics and intensities, increases in chitinase and β-1,3-glucanase activities and production of two phytoalexins, resveratrol and epsilon-viniferin. Application of laminarin on grapevine plants reduced infection by *B. cinerea* and *P. viticola* which could infect berries later, by about 55 and 75% respectively. By reducing the pathogen population on grapevine plants the incidence of the disease on berries was reduced considerably. The results indicate that activation of natural defense response using elicitors could be advantageously exploited as an effective strategy to protect grapevine plants against pathogens occurring at pre- and post-harvest stages (Aziz et al. 2003).

Crude extracts from several plant species have been reported to reduce infection of plants by microbial pathogens. Although the antimicrobial properties of the plant products have been demonstrated, only a few have the potential to induce systemic resistance to fungal and/or bacterial pathogens. Characterization of the biological inducers of resistance to disease and their potential to induce resistance in vivo are yet to be performed/proved.

5.1.2.2 Natural Products of Animal Kingdom

Chitosan derived from crab-shell has been tested for its ability to induce resistance to several fungal diseases infecting various crops. The biological activity of chitosan (CHN) (beta-1,4-linked glucosamine) oligomers is considered to depend on their binding to membrane receptors and on the molecular weight (MW) and degree of N-acetylation of the molecule (DA). Addition of chitosan (100 or 400 µg/ml) to the nutrient solution, protected cucumber plants against root rot caused by *Pythium aphanidermatum*. The host defense responses such as formation of structural barriers in root tissues and antifungal hydrolases such as chitinases, chitosanases and β-1,3-glucanase in both root and leaves were activated by the presence of chitosan, resulting in the restriction of pathogen development in treated plants (El Ghaouth et al. 1994). Chitosan applied as seed coating and substrate amendment in protected tomato plants against crown and root rot caused by *Fusarium oxysporum* f.sp. *lycopersici*. The chitosan treatment restricted fungal growth to the epidermis and cortex of roots of treated plants. Marked cellular disintegration characterized by enhanced vacuolation and ultimate loss of entire protoplasm of the pathogen were observed. The primary mechanism of resistance appears to be through formation of structural barriers at sites of attempted fungal penetration (Benhamou et al. 1994; Lafontine and Benhamou 1996). Chitosan was also effective in protecting tomato plants against late blight pathogen *P. infestans*. β-1,3-glucanase and chitinase genes were strongly induced, whereas PR-proteins, 1,3-hydroxy-3-methylglutaryl coenzyme A reductase, anionic peroxidase and PAL were weakly induced by chitosan treatment (Oh et al. 1998). Since chitosan is nontoxic to plants and biodegradable, it seems to be an acceptable alternative to chemical control strategy.

The CHN oligomers induced defense responses in grapevine leaves, as evidenced by an accumulation of stilbene phytoalexins, trans- and cist-resveratrol, ε-viniferins and piceids and a stimulation of chitinase and β-1,3-glucanase activities. CHN applied in combination with $CuSO_4$ strongly induced phytoalexin (resveratrol and their metabolites) accumulation. Induction of cist-resveratrol and cis-ε-viniferin was very responsive to $CuSO_4$. This activity of $CuSO_4$ in grapevine appears to have been recorded for the first time. These two phytoalexins were considered to be important markers of induced resistance in grapevine. The eliciting ability of CHN and $CuSO_4$ was associated with an induced resistance to gray mold disease caused by *B. cinerea* and downy mildew disease caused by *Plasmopara viticola* (Aziz et al. 2006).

5.1.2.3 Chemical Inducers

A wide range of inorganic and organic compounds has been tested in vitro for their efficacy to induce resistance in crops to diseases of economic importance. However, only very few of them have been demonstrated to have the potential for large scale application under field conditions.

Inorganic Compounds

Induction of resistance to a plant disease using phosphate salts was first demonstrated by Gottstein and Kuć (1989). Cucumber plants sprayed with phosphate solution developed resistance to anthracnose disease caused by *Colletotrichum lagenarium*. Application of 0.1 M phosphate salts on the upper surface of maize leaves induced systemic resistance to *Puccinia sorghi* causing rust disease with an additional advantage of stimulating the growth of phosphate-treated plants. Phosphates are considered to generate an endogenous SAR signal, because of calcium sequestration at the points of phosphate application (Reuveni et al. 1994). Similar enhancement of resistance of cucumber plants to powdery mildew pathogen *Podosphera xanthii* was also observed following application of phosphates. The activities of peroxidase and β-1,3-glucanase were increased in the protected uninoculated top leaves of cucumber plants (Reuveni et al. 1997). These results indicate the possibility of exploiting the induction of SAR for the protection of field crops against microbial pathogens by using inexpensive chemicals without obvious adverse effects on crop development. Phosphate-mediated resistance in cucumber was shown to be associated with localized cell death, preceded by a rapid generation of superoxide and H_2O_2. In addition, local and systemic increases in levels of free and conjugated salicylic acid following phosphate application were also recorded (Orober et al. 2002).

The effectiveness of induced resistance for protecting harvested produce against diseases in storage has also been investigated. Vacuum infiltration of calcium (Ca) into apple fruits was found to be more effective than field application in protecting the fruits against gray mold disease (Conway and Sams 1983). Exogenous application of silicon (Si) as sodium metasilicate reduced the development of *Penicillium expansum* and *Monilinia fructicola* infecting sweet cherry fruit at 20°C. Treatment with Si induced significant increase in the activities of PAL, PPO and PO in sweet cherry fruit. In addition, the biocontrol efficacy of the yeast antagonist *Cryptococcus laurentii* was markedly increased, when it was combined with Si application (Qin and Tian 2005). In order to have a deep insight into the role of Si in protecting plants against pathogens, a complete transcriptome analysis of both control and powdery mildew (*Erysiphe cichoracearum*)- infected *Arabidopsis* plants with or without Si application was performed, using a 44 K microarray. Inoculation of plants with or without Si treatment, altered the expression of a set of nearly 4000 genes. Many of the up-regulated genes were defense-related, whereas a large proportion of down-regulated genes were involved in primary metabolism. Regulated defense genes included *R* genes, stress-related transcription factors, genes involved in signal transduction, the biosynthesis of stress hormones (SA, JA, ET) and the metabolism of reactive oxygen species (ROS). This study provided evidence that contradicts the role of Si in passive resistance offered against plant pathogens evidenced by earlier reports (Fauteux et al. 2006).

Enhancement of levels of resistance of many monocot plants to fungal pathogens has been observed, when the plants are grown in soils amended with Si. The resistance mediated by Si to rice blast disease (*Magnaporthe grisea*) was suggested

to be due to a mechanical barrier produced from Si polymerization in planta. The cytological and molecular features associated with resistance to *M. grisea* in compatible and incompatible interactions with rice cultivars supplied with Si were studied. Differential accumulation of glucanase, peroxidase and PR-1 transcripts were associated with limited colonization by *M. grisea* in epidermal cells of Si+plants of the susceptible cultivar M201. On the other hand, Katty, a resistant cultivar responded to an avirulent race of *M. grisea* through the development of a HR along with a strong induction of PR-1 and peroxidase transcripts independent of Si amendment. In Si+plants of M201, differential accumulation of transcripts from glucanase, peroxidase and PR-1 correlated with an inhibition of the spread of the fungus. The cytological observations indicated that the cytoplasmic granulation within epidermal cells appeared to be a reliable indicator of *R* gene-conditioned cell death associated with limited pathogen spread. The early autofluorescence in epidermal cells may be indicative of incompatible interaction. However, autofluorescence did not markedly differ at later stages in compatible and incompatible interactions. The results suggested an active participation distinct from single gene-defined resistance for Si in the defense of rice against *M. grisea* (Rodriguez et al. 2005).

Organic Compounds

Among a large number of organic compounds tested, salicylic acid (SA) and 2,6-dichloroisoniciotinc acid (INA) have been investigated extensively for their ability to induce systemic resistance in plants to diseases. They induce the same spectrum of SAR gene expression to levels comparable with those induced by biotic inducers (Métraux et al. 1991; Ward et al. 1991).

Treatment of *Arabidopsis* plants with INA followed by challenge with a virulent race of *Phytophthora parasitica* resulted in the lignification of intercellular hyphae and consequent retarded development of the pathogen (Sticher et al. 1997). Systemic resistance to pea powdery mildew (*Erysiphe pisi*) was induced by exogenous application of SA to leaves (Frey and Carver 1998). A positive relationship between induction of SAR by SA and INA and systemic induction of several SAR genes in *A. thaliana* was observed (Dempsey et al. 1993; Mauch-Mani and Slusarenko 1996). Application of INA on green bean (*Phaseolus vulgaris*) resulted in marked increase in the activities of chitinase and β-1,3-glucanase and accumulation of SA (Dann et al. 1996). If plants are conditioned by pretreatment with inducers of SAR, the systemically protected plants may be able to resist the pathogens more effectively. Pretreatment of parsley cell cultures with SA or INA markedly enhanced the ability of the cultures to produce coumarins, following treatment with an elicitor from *Phytophthora megasperma* f.sp. *glycinea* (Kauss et al. 1992). Treatment of *Arabidopsis* with INA followed by challenge with a virulent race of *Phytophthora parasitica* resulted in lignigication of intercellular hyphae and consequent retarded development of the pathogen (Sticher et al. 1997). INA may induce defense response prior to infection or potentiate defense responses following infection. INA, upon application, conditioned sugar beet plants leading to production of PR proteins at a faster rate, when infected by the fungal pathogen *Cercospora beticola* (Nielsen et al. 1994).

Similar effects were observed in cucumber-*Colletotrichum lagenarium* pathosystem (Siegrist et al. 1994).

Application of methyl salicylate (MSA) under field conditions reduced significantly decay caused by *Botrytis cinerea* in strawberry fruits. MSA was converted into SA and increased the activity of chitinase. Since MSA is one of the natural volatile compounds present in strawberry fruits, it can be safely applied as a non-toxic alternative to fungicide application (Kim and Choi 2002). During an incompatible interaction between *Bt-10* gene for common bunt resistance of wheat (*Triticum aestivum*) and an avirulent race of *Tilletia laevis*, a lipase gene and several wheat genes belonging to PR-protein classes 1, -2, -3 and -4 and -14 were differentially up-regulated (Lu et al. 2005). The responsiveness of the defense-related PR-genes to the exogenous application of SA, methyl jasmonate (MeJA), a biologically active derivative of JA, was studied in wheat seedlings on infection at 1, 2, or 3 weeks following emergence. The transcription levels of defense-related genes were assessed. Application of SA or MeJA coordinately activated transcripts of different groups of defence-related proteins and reduced common bunt infection. The transcripts of Chi 1, Chi 3, Chi 4, PR-1.1, PR-1.2, Glu 1 and a lipase were up-regulated in response to MeJA treatment and their expression was potentiated by infection by *T. laevis.* The highest transcript levels were associated with the 3-week seedling stage. Transcripts of Glu 2 responded almost exclusively to SA, were not potentiated by infection and the highest expression was observed in the 3-week seedling stage. On the other hand, transcripts of ns-LTP-1, ns-LTP-2 and Glu 3 were up-regulated in response to both SA and MeJ, not potentiated by infection and the highest transcripts levels were recorded in the 1-week seedling stage (Lu et al. 2006).

Perception of both general and specific pathogen-associated molecules triggers defense responses via signal transduction cascades and transcriptional activation of numerous genes. In chickpea (*Cicer arietinum*), putative genes potentially involved in defense responses, including the rapid synthesis of PR-proteins, presence of an oxidative burst and synthesis of putative cell-wall strengthening proteins and antimicrobial proteins have been identified (Coram and Pang 2006). The responses of three chickpea genotypes treated with defense signaling compounds SA, MeJA and aminocyclopropane carboxylic acid (ACC) to Ascochyta blight disease caused by *Ascochyta rabiei*, were investigated, using microarray technology. The observations were validated by quantitative RT-PCR assay. Of the 715 experimental microarray features, 425 (59.4%) were differentially expressed (DE) at least in one condition. According to treatment, 69%, 15.8%, and 57.6% were differentially expressed respectively by ACC, MeJ and SA. The coregulation of transcripts between treatments for each genotype with varying levels of disease resistance showed that large proportions of transcripts were independently regulated by ACC, MeJ or SA. Of the coregulated transcripts, the ACC-SA category contained the most for all genotypes lending support to the suggestion of cross-talk and overlap between signaling pathways (Salzman et al. 2005; Jalali et al. 2006). SA primarily regulated transcripts involved in the oxidative burst, PR-proteins and putative antimicrobial proteins. ACC treatment induced expression of a putative defensin in the resistant genotype (IC). In IC, potentially important transcripts were regulated by all three treatments,

suggesting that signaling pathways mediated by ACC/MeJ/SA may be involved in the IC response to the fungal pathogen. In the moderately resistant genotype (FL), many of the *A. rabiei* induced transcripts were down-regulated by the treatments. In the susceptible genotype (LA), of the *A. rabiei*-induced transcripts, many resembled PR-proteins that were induced by SA treatment only, suggesting the operation of SA-mediated defense response in the susceptible genotype (Coram and Pang 2007).

The product of *MPK3* gene in *Arabidopsis* participates in disease resistance mediated by the MAP kinase cascade. The expression of *MPK3* was induced by inoculation with fungal pathogen *B. cinerea*, leading to induction of resistance to the disease (Asai et al. 2002). The *MPK3* gene was reported to be induced by treatment with signaling compounds that induce defense gene expression (Schenk et al. 2000). But the molecular mechanism involved in the *MPK3*-mediated pathway is unclear. In order to understand the molecular mechanism of *MPK3 g*ene expression, the *MPK3* promoter was fused to the firefly luciferase gene to create a real-time monitoring system for regulated gene expression in planta. The fusion gene *MPK3::Fluc* was inserted into a transformation vector and then introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation procedure. The transgenic *Arabidopsis* plants harboring *MPK::Fluc* were inoculated with *B. cinerea*. Induction of *MPK3* promoter activity was detected in the inoculated leaf as an increase in the Fluc light emission by 24 h after inoculation. The transgenic *Arabidopsis* plants harboring *PR-1a:Fluc* could be used to detect the induction of the PR-1a expression by SA treatment. The induction of PR-1a in the leaves inoculated with *B. cinerea* could be detected at 48 h after inoculation, reaching higher levels at 72 h after inoculation. Systemic induction of the fusion genes occurred following challenge by the fungal pathogen (Tanaka et al. 2006).

The bioefficacy of plant defense activators such as benza (1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ether (BTH) and acibenzolar-S-methyl (ASM, a derivative of BTH) and DL-β-aminobutyric acid (BABA) in protecting crop plants against diseases by inducing resistance in treated plants has been assessed. Induction of resistance by BTH in several crops to different diseases has been demonstrated. Induction of systemic resistance by BTH in wheat to the powdery mildew pathogen *Blumeria graminis* f.sp. *tritici (Bgt)* was reported by Stadnik and Buchenauer (2000). The white mold and gummy stem blight diseases could be effectively reduced by inducing resistance in melons using BTH (Buzi et al. 2004). Application of BTH on strawberry plants delayed the development of gray mold disease on harvested fruits caused by *B. cinerea* and increased the storage life of the fruits too (Terry and Joyce 2000). The severity of dry rot disease caused by *Fusarium semitectum* in inoculated potato tuber was reduced by BTH treatment of tubers. The activity of β-1,3-glucanase in leaves, stem, tubers and stolons of BTH-treated potato plants was increased (Bokshi et al. 2003).

The effect of root treatment with BTH was examined in papaya plants challenged with *Phytophthora palmivora*. The activities of chitinase and β-glucanase were enhanced in treated papaya plants. The *NPR1* gene (*CpNPR1*) was isolated by homology to a conserved region of *Arabidopsis* and tobacco *NPR1* genes and four partial PR-1 (PR1) cDNAs from papaya identified by homology to region conserved

in all known *PR1* gene family members. The CpPR1b and CpPR1d were induced by BTH. The CpPR1d with highest level of homology to tobacco PR1a was considered as a convenient marker of SAR induction in papaya (Zhu et al. 2003). In a later investigation, 25 additional papaya gene showing elevated systemic expression at 3 d after BTH treatment were identified by suppression subtraction hybridization and confirmed and quantified by northern blots and quantitative RT-PCR. The induced genes of papaya included several PR genes and two genes with likely involvement in altering cell wall porosity and lignification. In addition, six genes with potential roles in establishing reducing conditions following the oxidative burst were induced too. The results suggest that the regulation of cellular redox status may play a role in SAR induction in papaya (Qiu et al. 2004).

The responses of two rice cultivars Tetep [resistant to *Magnaporthe grisea* (*Mg*) and *Cochliobolus miyabeanus* (*Cm*), causing blast and brown spot diseases respectively] and Nakdong (susceptible to both pathogens) to treatment with BTH and the conidial germination fluid (CGF) of *Cm* were assessed. Treatment with CGF induced rapid cell death in both cultivars, possibly due to the phytotoxins produced by *Cm*. Pretreatment with CGF induced significant resistance to *Mg* in cv. Nakdong, but not to *Cm*. Likewise, BTH significantly enhanced resistance to only *Mg*, but it failed to protect rice cv against *Cm*. Enhancement of resistance of cv. Nakdong to *Mg* by BTH or CGF treatment was correlated with more rapid induction of three monitored PR genes. Expression patterns of three PR genes suggested that the rice defense mechanisms against rice brown leaf spot disease are distinct from those against blast disease and their expression patterns in response to *Cm* infection were nearly identical in both susceptible and resistant cultivars. Rice appears to employ distinct mechanisms for its defense against *Mg* versus *Cm* (Ahn et al. 2005).

In rice, four genes *OsBIERF1* to *OsBIERF4* [*Oryza sativa* benzothiadiazole (BTH)-induced ethylene responsive transcriptional factor (ERF)] were identified. The expression patterns of *OsBIERF* genes in rice seedlings (cv. Yuanfengzao) after infection with rice blast pathogen *M. grisea* were studied to elucidate the involvement of BTH-induced rice defense responses. Expression of *OsBIERF1, OsBIERF3* and *OsBIERF4* was induced by treatments with BTH and SA, capable of inducing disease resistance response in rice. In the BTH-treated rice seedling, the expression of these three genes was further induced by infection with *M. grisea* compared with water-treated control seedlings. In the incompatible interaction, *OsBIERF1* and *OsBIERF3* were activated, but not in compatible interaction between rice and *M. grisea*. The results suggest that OsBIERF proteins may participate in different signaling pathways that mediate disease resistance responses (Cao et al. 2006).

Phospholipases are the enzymes hydrolyzing phospholipids and they are divided into phopholipase A, C and D (PLA, PLC and PLD). PLD is the predominant family of lipases present in plants. The phospholipase network is considered to be involved in mechanisms of plant systemic responses to pathogen attack. The involvement of PLC signaling was suggested during pathogenic interactions with plants. *OsPI-PLC1* expression was shown to be induced by chemical inducers SA, JA, MeJ and BTH. These treatments enhanced resistance to rice blast disease caused by *M. grisea* (Song and Goodman 2002). In a later investigation, the involvement of both phopholipase C

(PLC, EC 3.1.4.11) and D (PLD, EC 3.1.4.4) in the early responses of *Brassica napus* plants to the treatment with SA, MeJ and BTH was determined. Rapid activation (within 0.5 – 6 h treatment) of in vitro activity level was found for phosphatidyl inositol 4,5-biphosphate (PIP_2)-specific PLC (PI-PLC) and three enzymatically different forms of PLD. The results indicated that phospholipases may be involved in very early processes leading to systemic responses in plants and they are possibly initially activated on posttranscriptional level (Profotová et al. 2006).

Acibenzolar-S-methyl (ASM) has been shown to effectively induce resistance to diseases affecting some plant species. ASM-mediated systemic priming of PAL, chitinase activity and phytoalexin accumulation in cowpea occurred resulting in enhanced resistance to anthracnose caused by *Colletotrichum destructivum* (Latunde-Dada and Lucas 2001). In cucumber plants treated with ASM, *pal* gene was systemically primed following inoculation with *C. orbiculare* (Cools and Ishii 2002). ASM was found to be effective in reducing scab disease of Japanese pear caused by *Venturia naushicola* in the field, though several treatments with ASM were required (Brisset et al. 2000; Ishii et al. 2002). The tripartite interaction among Japanese pear-ASM-*V. naushicola* was studied to understand the molecular mechanism involved in the induction of resistance, since ASM did not have any antifungal activity. The defense responses induced by ASM included transcripts encoding PGIP that were highly and transiently promoted after scab inoculation of plants pretreated with ASM. Enhancement of PAL activity, SA accumulation and induction of several PR-proteins in ASM-treated plants suggested that resistance triggered by ASM may be SA-independent (Faize et al. 2004). The effects of pretreatment of *Brassica napus* (winter oilseed rape) with ASM or menadione sodium bisulphate (MSB) (a water soluble addition compound of vitamin K3), in addition to a biological inducer were assessed. Both chemical and biological inducers delayed symptom expression due to *Leptosphaeria maculans* causing Phoma stem canker (black leg) disease. The chemical inducers could trigger local and systemic resistance in the treated leaves and the ones far away respectively (Liu et al. 2006).

Compatible and incompatible (nonhost) interactions of barley powdery mildew pathogen *Blumeria gramins* f.sp. *hordei* (*Bgh*) with barley require common genetic and mechanistic elements of plant defense. By using macroarrays based on cDNAs derived from epidermal peels of plants pretreated with ASM, the expression of barley transcripts in the early host interaction with *Bgh* or the nonhost pathogen *B. graminis* f.sp. *tritici* (*Bgt*) respectively was compared. Spots (102) corresponding to 94 genes on the macroarray that gave significant *Bgh*-responsive signals early stage of infection (12- 24 h) were identified. Majority of up-regulated genes were defense-related whereas the majority of down-regulated genes were associated with house-keeping functions (Eichmann et al. 2006).

5.2 Induction of Resistance to Bacterial Diseases

Systemic acquired resistance (SAR) has been demonstrated as a plant immune response which can be induced after a local infection leading to conferment of

resistance throughout the plant to a broad spectrum of pathogens. Induction of SAR follows accumulation of the endogenous SA signaling molecule, which activates gene expression mediated by the master regulator protein NPR1 (non-expressor of PR genes 1, also known as NIM1). This NPR1 protein is translocated into host cell nucleus after exogenous application of SA and it interacts with TGA transcription factors to mediate gene expression. A massive build up of PR proteins occurs in the vacuoles and the apoplast, during development of SAR. The dramatic enhancement cannot be attributed to the basal activity of the protein secretory pathway alone. Hence, a coordinated up-regulation in the protein secretory machinery may be required to account for proper folding modification and transport of PR proteins (Wang et al. 2005). The endoplasmic reticulum (ER)-resident gene *BiP* was induced before the accumulation of PR-1 (Jelitto-Van Dooren et al. 1999). In order to provide genetic evidence for the requirement of up- regulation of the secretion-related genes for SAR, knockout mutants in five secretion-related genes were investigated. The T-DNA insertions in *BiP2, DAD1* and *SEC61*α compromised the plant's ability to efficiently secrete PR-1 after treatment with BTH. The reduction in PR protein secretion had a direct bearing on the impaired resistance to *Pseudomonas syringae* pv. *maulicola* ES4326. In double mutants secretion of PR1 was less compared with single mutants and consequent greater growth of the pathogen (Wang et al. 2005).

Molecular events triggered by the primary infection process play a critical role in SAR initiation. *Arabidopsis* candidate genes up-regulated by SAR-inducing pathogens at inoculation sites, as indicated in microarray experiments, were studied. A gene that was up-regulated in *Arabidopsis* leaves inoculated with avirulent or virulent strains of *P. syringae* pv. *maulicola* (*Psm*) showed homology to falvin-dependent monooxygenases (FMO) and was designated *FMO1*. An *Arabidopsis* knockout line of *FMO1* was found to be fully impaired in the establishment of SAR triggered by avirulent (*Psm avrRpm*) or virulent (*Psm*) bacteria. Loss of SAR in the *fmo1* mutants was accompanied by the inability to initiate systemic accumulation of SA and systemic expression of diverse defense-related genes. The *fmo* 1 mutation did not alter local disease resistance level significantly toward virulent or avirulent strains of *Psm* in native plants. *FMO1* expression was also systemically induced upon local infection by *Psm*. Such a systemic up-regulation could not be detected in the SAR-defective SA pathway mutants *sid2* and *npr1*, as well as in the defense mutant *ndr1*, indicating a close correlation between systemic *FMO1* expression and SAR establishment (Mishina and Zeier 2006).

Pseudomonas chlororaphis O6, a PGPR has been shown to induce resistance in tobacco against the soft rot pathogen *Erwinia carotovora* subsp. *carotovora (Ecc*) and *P. syringae* pv. *tabaci* (*Pst*) causing wild fire disease. The extracellular compounds produced by *P. chlororaphis* were purified to identify the bacterial determinants involved in induction of disease resistance. Based on the results of HPLC and nuclear magnetic resonance mass spectrophotometry, the active compound was identified as 2R, 3R-butanediol. This compound induced systemic resistance to *Ecc* SCC1, but not to *Pst.* Treatment of tobacco with the volatile 2R, 3R-butanediol enhanced aerial growth which was also observed during colonization by this bacterial species. The global sensor kinase GacS of *P. chlororaphis* O6 was found to

be a key regulator for induced systemic resistance against *Ecc* through regulation of 2R, 3R-butanediol production. This report appears to be the first one attributing the induced resistance to 2R, 3R-butanediol, a fermentation product and to the sensor kinase GacS for its production (Han et al. 2005).

5.2.1 Biotic Inducers

Inducing resistance in plants to diseases using biological inducers may be preferable because of their ecofriendly nature. Different species of *Trichoderma* have been demonstrated to be effective biocontrol agents in controlling crop diseases and they are known to act directly by producing toxic compounds and/or indirectly by inducing resistance to diseases caused by microbial pathogens. *T. asperellum* (*Ta*) (= *T. harzianum*) is able to penetrate the roots of cucumber seedlings and colonize the epidermis and outer root cortex (Yedidia et al. 1999). Root inoculation with *Ta* has been effective in protecting plants against several pathogens (Harman et al. 2004). Colonization of roots by PGPRs induces ISR in many pathosystems and this form of induced resistance requires components of JA signaling pathway followed by the ethylene signaling pathway (Pieterse et al. 2003). The involvement of the plant signal molecules such as JA, ET and SA in the development of ISR following inoculation of cucumber roots with *Ta* was examined. The protective effect of *Ta*-mediated plant resistance against angular leaf spot pathogen *Pseudomonas syringae* pv. *lachrymans (Psl)* was observed due to both the reduction of bacterial multiplication in leaves and the number of necrotic lesions formed on leaves. There was no direct antagonistic activity of *Ta* on *Psl,* indicating the protective effect of *Ta* was due to plant-mediated phenomenon. Analysis of signal molecules involved in the defense mechanisms and application of specific inhibitors indicated the involvement of JA and ET in the protective effect of *Ta* against *Psl.* The gene expression determined with real-time RT-PCR assay revealed that *Ta* modulated expression of genes involved in the jasmonate/ethylene signaling pathways (*Lox1, Pal1, ETR1* and *CTR1*) in cucumber plants. The main signal transduction pathway through which *Trichoderma*-mediated ISR was activated by using JA and ET as signal molecules, as revealed by the involvement of several JA/ET pathway-related genes. The results also indicated that the *Trichoderma*-induced state sensitized the plant to respond more efficiently to subsequent pathogen attack. The sensitization is apparent from both the reduction in disease symptoms and the systemic potentiation of the PR genes, Chit 1, β-1,3-glucanase and peroxidase. The wide spectrum of this potentiated gene expression suggests its crucial role in ISR mediated by *Trichoderma* spp. (Shoresh et al. 2005).

The protection level against tomato bacterial leaf spot diseases caused by *Xanthomonas euvesicatoria* 110c was significantly enhanced by using a light sphagnum peat mix inoculated with *Trichoderma hamatum* 382, although this BCA did not colonize aerial plant parts. High density oligonucleotide microarrays were used to have a better understanding of the mechanism of induction of resistance by *T. hamatum*

382 in tomato. The expression patterns of 15,925 genes in leaves prior to inoculation with bacterial pathogen were determined. The expression of genes in tomato leaves was consistently modulated by the BCA. Forty one of 45 differentiated expressed genes could be assigned to at least one of seven functional categories associated with biotic or abiotic stress and metabolism of RNA, DNA and protein. Four extensin and extensin-like proteins were induced. However, no significant changes in the principal markers of SAR expression was evident (Alfanao et al. 2007).

In addition to biocontrol agents, bacteriophages and compounds have been tested for their ability to induce resistance in treated plants against diseases caused by bacterial pathogens. Two bacterial strains *Bacillus pumilus* B122 and *Pseudomonas fluorescens* B130, two SAR inducers, harpin (Messenger, Eden Bioscience Corp. Bothell, WA), ASM and an unformulated bacteriophage (phage) mixture (Agriphage, OmniLytics Inc., Salt Lake City, UT) were evaluated for their efficacy in protecting tomato plants against bacterial spot disease caused by *X. campestris* pv. *vesicatoria (Xcv)*. ASM prevented the development of typical symptoms. However, necrotic spots typical of HR were produced on the leaves of plants treated with ASM alone. Electrolyte leakage and bacterial population assessment showed that ASM-treated plants responded to *Xcv* inoculation by eliciting an HR. Application of bacteriophages in combination with ASM suppressed visible HR, providing very effective control of the disease. It is possible to enhance the effectiveness of disease management by proper integration of available options (Obradovic et al. 2005).

5.2.2 Abiotic Inducers

Benzothiadiazole (BTH, Syngenta) was reported to protect apple from fire blight disease caused by *Erwinia amylovora (Ea).* BTH did not show any direct toxic effect on *Ea* in agar plates consistent with BTH being a SAR inducer. BTH was able to provide protection to Jonathan apple under field conditions and also to Golden Delicious seedlings, scions and trees following artificial inoculation (Brisset et al. 2000; Maxson-Stein et al. 2002). The effect of BTH was shown to be associated with increased gene expression and accumulation of defense-related enzymes. Transcripts of three PR-related genes PR-1, PR-2 and PR-8 were strongly induced by high concentrations of BTH in Jonathan seedling (Maxson-Stein et al. 2002). Peroxidase and β-1,3-glucanase activities (PR-2) in BTH treated Golden Delicious seedling were observed. Systemic stimulation of the enzymatic activities was also seen later (Brisset et al. 2000). Application of BTH on 2-year old pear plants protected them from fire blight both in terms of disease incidence and severity. Although the treated plants challenged with *Ea* harbored viable pathogen cells, as revealed by PCR-base detection, little or no symptoms of the disease could be seen at 6 months after inoculation. Expression of a member of the PR-1 family in pear plants treated with BTH and SA and subsequently inoculated with *Ea* was found to be constitutive and unaffected by treatments, suggesting that molecules other than PR-1 may be required for induction of systemic resistance by BTH (Sparla et al. 2004).

The effectiveness of BTH (abiotic) and an avirulent strain of *P. syringae* pv. *maulicola (Psm)* (biotic) in inducing SAR in *Brassica napus* (canola) against virulent strains of *Psm* and the fungal pathogen *Leptosphaeria maculans* was assessed. Application of BTH enhanced resistance against virulent strains of *Psm* and *L. maculans* to a greater extent than localized preinoculation of plants with avirulent strain. Pretreatments with BTH and avirulent strain resulted in accumulation of PR genes including *BnPR-1* and *BnPR-2*, the levels of transcript accumulation being greater in BTH-treated plants. Development of SAR in *B. napus* plants expressing a bacterial salicylate hydroxylase transgene (*NahG*) that is known to metabolize salicylic acid (SA) to catechol was appreciably compromised. These plants accumulated only reduced levels of PR gene transcripts compared with nontransformed controls. The results indicate that SAR development in *B. napus* had many hallmarks of classical SAR including long lasting and broad host range resistance, association with PR gene activation and requirement of SA (Potlakayala et al. 2007).

5.3 Induction of Resistance to Viral Diseases

Induction of SAR was first demonstrated in tobacco cv. Samsun NN which reacted with the formation of local necrotic lesions when inoculated with *Tobacco mosaic virus* (TMV). The level of SAR is determined by the reduction in number and/or diameter of lesions following challenge inoculation. Production of PR proteins in tobacco inoculated with TMV was related to development of resistance (Ross 1961a,b; Gianinazzi 1983). The PR protein induced in cucumber by *Tobacco necrosis virus* (TNV) was a chitinase and the plant exhibited resistance also to the bacterial pathogen *P. syringae* pv. *lachrymans* (Métraux et al. 1988). PR proteins (PRs) mostly accumulate in the extracellular space or in the vacuole in the cells of tobacco. Systemic induction of PR proteins may be due to enormous increase in endogenous levels of SA. The increase in SA level paralleled PR-1 production in TMV- resistant Xanthi nc (NN) tobacco plants, but not in susceptible (nn) tobacco (Yalpani et al. 1991). The loss of HR at higher temperature (32°C) was found to be related to failure of SA accumulation, suggesting that SA may act as endogenous signal triggering events leading to SAR. The uninoculated amphidiploid hybrid of *Nicotiana glutinosa* × *N. debneyi*, highly resistant to TMV had high levels of PRs due to constitutive expression. Fluorescence, ultraviolet and mass spectral analyses indicated that the healthy hybrid leaves contained higher SA levels (30 folds) than that of Xanthi nc tobacco leaves which did not constitutively express PRs and comparatively less resistant to TMV. Following inoculation with TMV, a steep increase in SA levels in leaves of Xanthi nc was observed as against a marginal increase in the hybrid. However, a dramatic increase in SA contents of phloem exudates from the hybrid (500 folds) over that of Xanthi nc was recorded. Exogenous application of SA induced production of PR-1 protein in Xanthi nc tobacco. On the other hand, no detectable change occurred in the constitutive expression of high levels of PR-1 proteins in the hybrid. These results strongly indicate the regulatory role of SA in disease resistance and PR protein synthesis (Yalpani et al. 1993).

Salicylic acid (SA) induces a range of defense genes, especially those encoding the PR proteins. The mechanism of development of SA-mediated resistance to plant viruses is still unclear. However, it was reported that treatment with SA resulted in accumulation of PR proteins and TMV titer was reduced in directly inoculated leaves of tobacco. In addition, the ratio of genomic RNA to CP mRNA and the ratio of plus-to minus-sense RNAs were also affected by SA application (Chivasa et al. 1997), suggesting that SA-induced interference with the activity of TMV- RNA-directed RNA polymerase (RdRp). Salicylhydraoxamic acid (SHAM), an inhibitor of plant mitochondrial alternative oxidase (AOX) inhibited the delay in symptom expression in TMV-inoculated tobacco plants treated with SA. Further, the SA-induced SAR in tobacco plants with N gene was also inhibited (Chivasa et al. 1997). The defensive signal pathway appears to separate downstream of SA into (i) one branch leading to induction of resistance to TMV that is SHAM sensitive; (ii) other SHAM insensitive pathway leading to induction of antifungal and antibacterial mechanisms (Chivasa et al. 1997). In the case of *Potato virus X* (PVX), RNA accumulation in inoculated tobacco leaf tissue was reduced by SA treatment and resistance was also found to be dependent in the SHAM-sensitive signaling pathway. In contrast, symptom development in SA-treated and *Cucumber mosaic virus* (CMV)-inoculated plants was also delayed. This was not due to inhibition of replication, but rather to inhibition of systemic movement of the virus. SA-induced resistance to CMV was lost due to the action of SHAM. In the case of virus diseases, SHAM-sensitive signaling pathway seems to activate both resistance mechanisms: inhibition of long-distance movement and inhibition of virus replication as in TMV and OVX (Naylor et al. 1998).

5.3.1 Abiotic Inducers

Salicylic acid (SA) application on tobacco enhanced the resistance to CMV and the resistance was shown to be due to inhibition of systemic virus movement. Induction of resistance to CMV occurred via signal transduction pathway that may also be triggered by antimycin A, an inducer of the mitochondrial enzyme alternative to oxidase (AOX). In *A. thaliana* inhibition of CMV systemic movement was also induced by SA and antimycin A. In squash (*Cucurbita pepo*), SA-induced resistance to CMV was attributed to the inhibition of virus accumulation in directly inoculated tissue most likely through inhibition of cell-to-cell movement. Different host plant species may adopt markedly different approaches to tackle infection by the same virus. It is essential that adequate caution has to be exercised, while attempting to apply findings on plant-virus interactions from model systems to a wider range of host species (Mayers et al. 2005).

Application of SA delays the symptoms induced by PVX and CMV in tobacco and *Arabidopsis* plants (Naylor et al. 1998; Mayers et al. 2005). The effects of SA and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene biosynthesis on *Potato virus Y* (PVY) isolate N:O-induced systemic symptom expression

in tobacco plants were assessed. Treatment of tobacco seedlings with SA delayed virus-induced necrosis in stems by one to two days. SA, but not ACC, was able to suppress severity of symptoms on stems significantly. However, both compounds did not affect the recovery of plants from severe phase of the disease symptoms. SA enhanced peroxidase activity in stems and PR genes were activated in both stem and leaves of tobacco. The results suggested that SAR has a critical role in suppressing $PVY^{N:O}$-induced symptom development through SA-mediated and ET-independent pathways. Reduced replication/accumulation of the virus is reflected by the suppression of disease symptom in SA-treated plants (Nie 2006).

5.3.2 Biotic Inducers

The plant growth-promoting rhizobacteria (PGPR), as in the case of fungal and bacterial diseases, have been reported to induce systemic resistance to some virus diseases also. *P. fluorescens (Pf)* stain 89B-27 could induce systemic resistance to CMV in cucumber cv. Straight 8, leading to consistent reduction in mean numbers of symptomatic plants coupled with delay in symptom expression. No viral antigen could be detected in the asymptomatic plants throughout the experimental period (Raupach et al. 1996). The strains Pf1 and CHA0 of *Pf* were able to induce systemic resistance in rice against rice tungro disease, when these strains were applied as seed treatment, root dipping or foliar spray (Narayanasamy 1995). The *Pf* strain P3 does not synthesize SA naturally. Introduction of *pchBA* which encodes for biosynthesis of SA in *P. aeruginosa* into Pf strain 3, rendered it capable of SA production in vitro. Consequently the ability of the transgenic strain significantly improved in inducing systemic resistance in tobacco against TNV. Introduction of *pchBA* gene into *Pf* strain CHA0 increased its ability to produce SA in vitro. However, there was no increase in its efficiency of inducing systemic resistance in tobacco against TNV. Furthermore, the capacity of strains P3 and CHA0 to suppress the black root rot of tobacco was not improved due to the presence of pchBA (Maurhofer et al. 1998). Tomato plants were protected by the treatment with *Pf* against *Tomato spotted wilt virus* (TSWV). Enhancement of the activities of defense-related enzymes such as peroxidase and phenylalanine-ammonia lyase was observed in many crops treated with the PGPR strains (Narayanasamy 2006).

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Chapter 6
Molecular Biology of Biocontrol Activity Against Crop Diseases

Abstract As an ecofriendly strategy of crop disease management, use of biocontrol agents (BCAs) for protecting crops against diseases has attracted the attention of researchers, industries and consumers of agricultural and horticultural produce. Although several microbes have been shown to have the potential for use as BCAs, the satisfactory performance under field conditions has been proved only for a few of them. Molecular techniques have been applied to establish the identity and genetic diversity of microorganisms with potential biocontrol activity and to gather information on the genes required for and molecular determinants of the biocontrol activity of different BCAs. The studies on the molecular bases of the three-way interaction among the plant, BCA and pathogen have opened up the possibility of having a better understanding of how the BCAs search for the pathogen, talk to the host plant and survive in the environment.

Use of biocontrol agents (BCAs) is considered as a desirable crop disease management strategy alternative to chemical application due to ill effects of chemicals to the environment, consumers of agricultural produce and non-target organisms. Molecular techniques have accelerated the pace of research endeavors to gather information on various aspects of BCAs and their interaction with pathogens and plants. The BCAs can interact with the microbial pathogens by producing different toxic metabolites such as enzymes and antimicrobial compounds. They also have the ability to indirectly interact with pathogens by inducing resistance in plants to diseases caused by them. The foremost step in the study of molecular biology of the BCAs is establishing the identity of the BCAs with certainty at genus, species, subspecies or strain level. This will be essential for the reproducibility and credibility of the tests performed at various centers and for patenting and development of BCA-based products for large scale application.

6.1 Identification and Differentiation of Biocontrol Agents

Molecular techniques have been shown to be rapid and reliable in establishing the identity of microbial pathogens (Volume 1, Chapter 2). These techniques can

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be easily modified, if necessary, and applied for the identification of the biocontrol agents up to strain levels. Such precise identification is required, because the strains/isolates of one species of the fungus or bacteria may have widely varying biocontrol potential. Further, the biocontrol activity may also vary depending on the crop plant species, type of soil or environment available in different ecosystems.

6.1.1 Fungi as Biocontrol Agents

The importance of precise identification of the BCA is underscored by the example of *Trichoderma harzianum (Th*) which has been registered as a biocontrol agent for the control of several plant diseases (Chet and Inbar 1994). But *Th* is also known as the causal agent of green mold disease of mushroom (Ospina-Giraldo et al. 1999). In addition, some isolates of *Th* have been reported to produce the mycotoxin belonging to the trichothecene class which can cause serious ailments in humans and animals (Sivasithamparam and Ghisalberti 1998). The isolation of a putative trichodiene synthase (*tri5*) gene required for trichothecene biosynthesis was reported for the first time in *Trichoderma*. By using specific *tri5* primers for a PCR protocol, it was possible to detect the presence of *tri5* gene in the genomes of different *Trichoderma* spp., including *Th*, facilitating the selection of suitable *Th* strain for biocontrol activity (Gallo et al. 2004).

Attempts have been made to determine the relationship between functional group within *Trichoderma* spp. and their biocontrol activity based on a combination of physiological, biochemical (enzyme production) and molecular (ITS sequences) criteria. The efficacy of particular strains of *T. harazianum* depended on the intended target and the required functions for biocontrol. This study also highlighted the importance of selection of the most efficient strains for the targeted pathogen (Grondona et al. 1997). One of the mechanisms of biocontrol activity in *Trichoderma* spp. has been suggested to be the stimulation of defense-related responses (Howell et al. 2000). The influence of culture filtrates (CFs) from effective biocontrol strains of *T. virens* on the stimulation of defense-related compounds was investigated. The CFs from biocontrol-effective strains of *T. virens* significantly stimulated terpenoid levels in the water-and media-treated controls and the ineffective strain. SDS-PAGE analysis of the active material revealed the presence of several bands in the material from biocontrol-active strains and these bands were absent in the material from inactive strains (Fig. 6.1). After elution from the gel, four bands were shown to be involved in the stimulation of cotton terpenoid production. One of them cross-reacted with an antibody to the ethylene (ET)-inducing xylanase from *T. viride*, whereas another band (18-kDa) stimulated cotton terpenoid production significantly, in addition to an increase in peroxidase activity in cotton radicles (Hanson and Howell 2004).

Ampelomyces quisqualis is a hyperparasite of powdery mildew pathogens and one of the *A. quisqualis* isolates has been developed as a commercial biofungicide (AQ-10 Biofungicide, Ecogen Inc., Langhorne, Pa). As this isolate of *A. quisqualis*

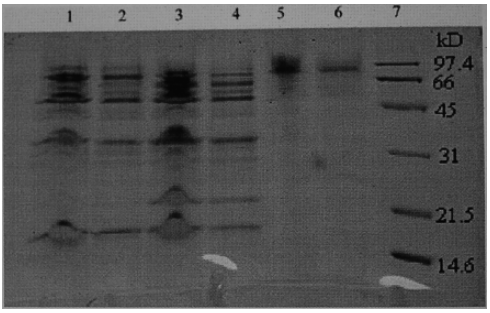

Fig. 6.1 SDS-PAGE analysis of culture filtrates (CFs) from effective and ineffective biocontrol strains of *Trichoderma virens*
Lanes 1 and 2: CFs from *T. virens* strain G6 (biocontrol effective); Lanes 3 and 4: CFs from *T. virens* strain G6-5 (biocontrol effective); Lanes 5 and 6: CFs from *T. virens* strain G6-4 (biocontrol ineffective); Lane 7: Molecular size markers (kD). (Courtesy of Hansen and Howell 2004; The American Phytopathological Society, St. Paul, MN, USA)

showed variations in biocontrol potential, it was essential to select the most effective one. Restriction fragment length polymorphism (RFLP) and sequence analysis of the rDNA ITS indicated high genetic diversity with *A. quisqualis* isolates which could not grouped into a single species (Kiss 1997; Kiss and Nakasone 1998). This study emphasized the need to apply precise technique to identify the isolate/strain of BCA that can provide effective protection against targeted pathogen. Furthermore, the diversity in fungal species has to be assessed (Volume 1, Chapter 3) for applicability of the candidate species/isolate to different hosts or ecological zones. Such assessment will ensure the efficacy of the BCA under varying conditions and minimization of risks of large scale introduction of inappropriate BCAs.

Identification of fungal antagonists based on morphological characteristics may lead to wrong notions or conclusions. For example, *Pseudozyma flocculosa*, an effective antagonist against powdery mildew diseases in greenhouse crops, was initially classified under the genus *Sporothrix* (Traquair et al. 1988). Since some species of *Sporothrix* such as *S. schenkii* are known human pathogens (Bennett 1990), the registration of *P. flocculosa* had to face the grave concern expressed by governing bodies. The physiological, biochemical and molecular studies established particularly based on sequence analysis of the larger nuclear subunit of rDNA that this fungal species should be reclassified under the genus *Pseudozyma* (Boekhout 1995). Later investigation using molecular markers rDNA for identification of *P. flocculosa* confirmed that *P. flocculosa* was genetically distant from *Sporothrix* and specific isolates could be selected for efficiency as biocontrol agents (Avis et al. 2001).

Maintenance of fungal strains to be used as BCA is essential to have a reliable source of authenticated fungal strains and to ensure consistency of performance of the BCA both in vitro and in vivo. The quality control test is required to allow monitoring of fungal isolates that are to be released into natural or cultural environments. Predominantly, DNA markers that allow authentication of strains and permit monitoring of contamination have been employed (Markovic and Markovic 1998).

In addition, potential mutations that affect the biocontrol efficiency of the BCA have to be detected as and when they occur (Becker and Schwinn 1993). The quality control test for *Trichoderma* is based on production of PCR fingerprints by using semi-random primers designed to primarily target intergenic, more variable areas in the genome (Bulat et al. 1998). Universally primed PCR (UP-PCR) employs single, 15 to 20-bp long primers that produce multiple amplification products without the need for information on DNA sequences of the test organism. This test can resolve more precisely similar *Trichoderma* strains, when compared with ITS ribotyping and RAPD procedures, because of generation of numerous fragments (60–100 per primer) which cover an important portion of the genome. The UP-PCR was considered to be powerful tool that could be employed for monitoring strains of interest as well as for identification of industrially important strains of *Trichoderma* (Lübeck et al. 1999).

Molecular markers have been shown to be useful for distinguishing strains of *Aureobasidium pullulans* which has been an effective biocontrol agent against several postharvest pathogens infecting citrus, grapes, apples and strawberries. Rapid identification and quantification of desired strains of the BCA is essential for monitoring the population levels at different periods and different ecological zones and also for evaluation of colonization and dispersal in the environment. The isolates of *A. pullulans* from the surfaces of several fruits and vegetables were subjected to molecular analysis by using arbitrarily primed PCR (AP-PCR). This investigation showed that there was high genetic variability within *A. pullulans* and the biocontrol potential of 41 isolates varied widely (Schena et al. 1999). RAPD technique was used for a preliminary screening of *A. pullulans* for genetic variability among 205 isolates. Consequently, the presence of a 1.3 kb fragment (L4) present only in isolates 47 and 633 was observed. Two SCAR primers and a 242-bp probe were generated based on the sequences of cloned L4 fragment. Both the SCAR primers and the riboprobe labeled with DIG were highly specific for L47. The SCAR primers (AP13 and AP14) consistently amplified a SCAR marker of approximately 150-bp from the genome of strain L47 up to the limit of 20 pg/µl. One of the SCAR primer AP 13 was modified to obtain a Scorpion primer for detecting a 150-bp amplicon by fluorescence emitted from a fluorophore through a self-probing PCR assay. This assay specifically recognized the target sequence of L47 strain over a number of other *A. pullulans* isolates in field-treated grape berry washings, the limit of detection being 10^5 cells per ml (10 folds greater sensitivity than CFU assay). In addition, this protocol was also useful to demonstrate the epidermis of sweet cherry fruits and to track it in the mesocarp (Schena et al. 2002).

The epiphytic yeast *Pichia anomala* strain K was selected for its high and reliable protective activity against *Botrytris cinerea* causing gray mold and *Penicillium expansum* causing blue mold disease of apple respectively (Jijakli et al. 1993). For monitoring of strain K, a specific method capable of quantifying the BCA population and distinguishing it from the indigenous microflora had to be developed. The protective effect of strain K was closely related to colonization on apple fruit surface. Hence, evaluation of the ecological fitness of strain K after treatment on apples was found to be critical for the interpretation and prediction of its biocontrol

efficacy in relation to several parameters such as application method and environmental conditions of storage rooms. A quantitative-competitive PCR (QC-PCR) method with enzyme-linked oligosorbent assay (ELOSA) was developed to monitor *P. anomala* strain K. The ELOSA method involves the binding of both target and internal standard (IS) sequences in the same microwell using a single capture probe followed by differential binding of specific detection probes for each amplification product. The combination of PCR and ELOSA was found to be highly specific, based on primers and DNA probe hybridization specificity. The QC-PCR-ELOSA based on the sequence-characterized amplified region (SCAR) marker (262-bp) was developed for monitoring strain K population. It was possible to quantify the number of yeast cells with an acceptable level of accuracy within the range of 10^3–10^6 yeast cells. This procedure was very specific and sufficiently accurate to monitor strain K populations within a short period of time compared to plating technique (Pujol et al. 2004). In a later investigation, a real-time PCR assay using a 3′-Minor Groove Binding (MGB) probe was developed for specific detection and monitoring of another yeast species *Candida oleophila* strain O effective against *B. cinerea* and *P. expansum* on harvested apples. The application of RAPD technique followed by reproducible SCAR amplifications allowed the identification of a 244-bp fragment in strain O and three other strains. But a 3′-MGB probe specifically matched a region of O strain sequence. This specific detection method was applied to monitor strain O population rapidly (Massart et al. 2005).

Role of exo-β-1,3-glucanase in the protection of apple fruits by the antagonistic yeast *Pichia anomala* strain K against *B. cinerea* was investigated. The genes *PAEXG1* and *PAEXG2* encoding exo-β-1,3-glucanase were separately and sequentially disrupted. These genes were inactivated sequentially with the unique *URA3* marker gene. The efficiency of the mutants was markedly reduced as reflected by the calculated protection level dropping from 71% (wild-type strain) to 8% (mutated strain) in wounded apple fruit under certain experimental conditions. The results suggested that the contribution of exo-β-1,3-glucanase of *P. anomala* to biological control may be masked by other modes of action under certain conditions, indicating that principal mode of action may vary depending on the storage conditions and physiological stage of apple fruit (Friel et al. 2007).

6.1.2 Bacteria as Biocontrol Agents

Bacteria belonging to the genera *Bacillus* and *Pseudomonas* have been reported to provide protection to crop plants against diseases caused by microbial pathogens. The gene fragment associated with rhizosphere competence in different biocontrol strains of the plant growth-promoting rhizobacteria (PGPR) *Pseudomonas fluorescens (Pf),* has been identified (Mavrodi et al. 2002). In another study, a sequence homologous to a bacteriocin was identified and this finding led to the discovery of a population of *Pf* that could produce bacteriocins and hence antagonistic to other isolates. The strains with this sequence competed with other organisms in wheat

rhizosphere and established an effective *Pf* population (Validov et al. 2005). *Bacillus subtilis* is ubiquitously present in agricultural soils and some strains are able to suppress the development of plant pathogens, resulting in marked improvement in plant health (McSpadden Gardener 2004). The genetic basis of biocontrol activity of *B. subtilis* strains is not clearly known. By employing suppressive subtractive hybridization (SSH), the genomes of GB03 and QST713 strains of *B. subtilis* were screened for strain-specific genetic elements that could be related to biocontrol activities. Cyclic lipopeptides (CLPs), antibiotic compounds synthesized nonribosomally by large peptide synthetases, contribute to the biocontrol activities of many *B. subtilis* strains. Genetic disruption of the CLP iturin biosynthesis reduced the suppression of *Rhizoctonia solani* by the strain RB14 (Tsuge et al. 2001). Analyses of the mutants of *B. subtilis* strain FZB42 indicated that fengycin and bacillomycin D acted synergistically to inhibit the growth of *Fusarium oxysporum* in vitro (Koumoutsi et al. 2004).

Natural disease suppression in soils is known for a long time and such soils have been the source of several antagonists. The occurrence of disease suppressiveness in soil-less systems is a poorly understood phenomenon. The loss of suppression after sterilization could be partly restored by addition of original microflora. The microorganisms in used rockwood slabs that were responsible for suppression of *Pythium aphanidermatum* causing damping-off disease in cucumber were analyzed. The community structure and diversity were determined by PCR – denaturing gradient gel electrophoresis (DGGE) of total community DNA. The canonical correspondence analysis (CCA) was used to perform the statistical analysis of the PCR-DGGE profiles and their correlation with disease suppressiveness. The microbial populations were examined for their composition and diversity in relation to disease suppression. PCR-DGGE profiles of the bacterial and actinomycete populations differed among treatments. The bands from the actinomycetes specific-PCR-DGGE patterns which were sequenced, showed high similarity (96–100%) with sequences in database of the several genera. Of these microbes, *Streptomyces* spp. was predominant. Different species of *Streptomyces* are being used as primary components of commercial products such as Mycostop (Verdere, Finland), Rhizovit (Prophyta, Germany) and Actinovate (Natural Industries Inc., USA). Several *Streptomyces* strains were isolated from used rockwood slabs. As the population size of the culturable filamentous actinomycetes was strongly correlated with disease suppressiveness, the actinomycetes may play an important role in suppression of *Pythium* spp. (Postma et al. 2005).

In order to develop molecular markers for identification and characterization of *B. subtilis*, attempt was made to establish association of several genetic elements with biological control of plant pathogens by this BCA. Over 60,000-bp of genomic sequences obtained from strains of *B. subtilis* (QST713 and GB03) used in two different biocontrol products were characterized. The sequences of 149 subtracted fragments shared a high degree of similarity to sequences found in multiple *B. subtilis* strains with demonstrable biocontrol activities. Oligonucleotide primers specific to five of these nine genes (*bmyB, fenD, ituC, srfAA* and *srfAb*), targeted the genes involved in antibiotic production and four additional genes (*yndJ, yngG,*

bioA and a hypothetical ORF) not previously associated with biological control. The PCR-based assays developed in this study may be employed to identify new biocontrol strains of *B. subtilis*. The sequence analyses showed that all isolates containing the markers were genetically distinct. The commercially successful strains GB03, QST713 and MB1600 contained the genetic markers identified in this study, indicating the practical utility of these markers for selecting novel and more efficient strains of *B. subtilis*. Strains that scored positive for the amplifiable markers generally were more effective in inhibiting the growth of *R. solani* and *Pythium ultimum* than the strains that lacked the markers (Joshi and McSpadden Gardener 2006).

6.2 Molecular Basis of Biocontrol Potential

An understanding of the mechanisms of action of BCAs is considered as the basic requirement for practical application of effective ones, because this information allows a more rational mode of production and formulation enhancing the effectiveness of selected BCA species/strains. Microbes capable of protecting plants either directly or indirectly contain genes encoding enzymes, antibiotics and resistance-inducing compounds. The toxic metabolites may suppress the development of the pathogens, whereas some compounds may function as elicitors of resistance to diseases in plants treated with BCAs.

6.2.1 Fungal Biocontrol Agents

Among fungi that have the potential for use as BCAs, *Trichoderma* spp. appears to be the leader, as reflected by the availability of more than 50 different *Trichoderma*-based agricultural products registered in different countries for use to protect and improve yield of vegetables, ornamentals and fruit trees (Woo et al. 2006). Strains of *Trichoderma* secrete different enzymes and antimicrobial compounds. *T. harzianum* (*Th*) produces trichodermin and a small peptide that can inhibit *Rhizoctonia solani* which in turn, secretes a coumarin derivative capable of inhibiting the mycelial growth of *Th*. However, the antimycotic compound produced by *Th* was more inhibitory even at low concentration than those produced by *R. solani* (Bertagnolli et al. 1998). *T. harzianum* acts on *B. cinerea* first by antibiosis leading to cell death, followed by dissolution of cells by producing chitinolytic enzymes (Bélanger et al. 1995). In addition, this BCA can adversely affect the pathogenic potential by counteracting activities of polygalacturonase (PG), pectin methylesterase (PME) and pectate lyase (PL) of *B. cinerea*, resulting in reduction in disease severity (Zimand et al. 1996). The enhanced levels of endochitinase detected in the rhizosphere of soybean were due to the activities of *Th* which effectively suppressed *R. solani* (dal Soglio et al. 1998).

Attention of researchers was focused on the role of cell wall-degrading enzymes (CWDEs) and antibiotics in mycoparasitism or antagonism of BCAs against plant

pathogens. However, studies based on mutants and targeted gene disruption, were unable to clearly identify enzymes required for biocontrol independently of the strain and the system, because of the redundancy in the genome of *Trichoderma* of CWDE-encoding genes. *T. harzianum* strain P1 effective against foliar and postharvest pathogens such as *B. cinerea* secretes several chitinolytic enzymes including N-acetyl-β-glucosaminidase (CHIT 72), chitin 1,4-β-chitbiosidase (CHIT 40) and a single 42-kDa endochitinase (CHIT 42). The strain P1 was genetically modified by targeted disruption of the single copy *ech42* gene encoding for the secreted CHIT 42. The stable mutants lacked the *ech42* transcript, the protein and endochitinase activity in culture filtrates. Other chitinolytic and glucanolytic enzymes expressed during mycoparasitsm were not affected by disruption of *ech42*. The mutant was as effective as P1 strain against *Pythium ultimum*, whereas its effectiveness against *B. cinerea* on bean leaves was reduced by 33%. However, the endochitinase-deficient mutant was more effective against the soilborne pathogen *R. solani* than the wild-type P1 strain. The results indicated that the biocontrol activity of *T. harzianum* may depend on the fungal pathogen involved in the interaction (Woo et al. 1999).

Genetic studies to have an insight into the antagonistic mechanisms of the BCA *Pichia anomala* strain K and its segregants were taken up. The gene encoding for exo-β-1,3-glucanase was isolated and sequenced. Disruption of this gene resulted in abolition of all extracellular exo β-1,3-glucanase activity in vitro and in situ (Jijakli and Lepoivre, 1998). The purified exo-β-1,3-glucanase from culture filtrate (CF) of strain K (paexg2) inhibited the germtube growth of *B. cinerea* strongly, in addition to inhibition of conidial germination and induction of morphological changes. The exo-β-1,3-glucanase activity detected on apples treated with strain K was similar to paexg2 in several properties. Two genes *PAEXG1* and *PAEXG2* coding for exo-β-1,3-glucanase were identified in the genome of strain K using PCR with degenerate primers designed on the basis of conserved amino acid region and on the N-terminal sequence of paexg2 (Grevesse et al. 1998a,b). The segregation of *PAEXG1* and *PAEXG2* alleles in haploid segregants indicated that there was no relationship between exo-β-1,3-glucanase activity in vitro and their biocontrol potential against *B. cinerea* in apples (Grevesse et al. 1998b). The *PAEXG2* gene encoding exo-β-1,3-glucanase was isolated from *P. anomala* strain K and the gene product was characterized. *PAEXG2* codes for an acidic protein consisting of 427 amino acids with MW of 45.7-kDa. Disruption of *PAEXG2* gene by insertion of the URA3 marker gene encoding orotidine monophosphate decarboxylase resulted in a reduction in biocontrol potential, as well as in reduced colonization of wounds in apples. Disruption of *PAEXG2* led to loss of all detectable exo-b-1,3-glucanase in vitro and in situ. However, the biocontrol activity in wounded apples against *B. cinerea* was not affected by disruption of this gene, indicating that the biocontrol activity of strain K did not depend on the production of exo-β-1,3-glucanase (Grevesse et al. 2003).

In a later investigation, the genes potentially involved in the biocontrol activity of *P. anomala* strain Kh 5 were identified using cDNA-AFLP analysis. Thirty five primer pairs were employed in AFLP amplification resulting in formation of a total of more than 2,450 bands derived from the mRNA of strain Kh 5 grown in the presence of cell walls (CW) of *B. cinerea.* Eighty six bands corresponded to

genes upregulated in the BCA grown in the presence of CW, compared with BCA without CW in the medium. The real-time RT-PCR assay confirmed the differential expression of the BCA in the presence of pathogen CW. Following normalization of the results of RT-PCR using appropriated house-keeping gene *G2*, eleven genes showed marked increase in expression in the presence of CW of *B. cinerea*. The overexpressed genes exhibited homologies to yeast genes with various function, including β-glucosidase transmembrane transport, citrate synthase and external amino acid sensing and transport. Some of these functions had a bearing on the biocontrol potential of *P. anomala* strain Kh 5 (Massart and Jijakli 2006).

6.2.2 Bacterial Biocontrol Agents

The bacterial biocontrol agents, as in the case of bacterial pathogens, respond to the phyllosphere/rhizosphere environment prior to initiating the biocontrol activities per se. The presence of a putative copper-transporting P1-type ATP-ase (CueA) in the genome of PGPR, *Pseudomonas fluorescens* (*Pf*) SBW25 has been reported. On reaching the plant surface, CueA was found to be induced in *Pf*. By using, a chromosomally-integrated *cueA-'lacZ* fusion, induction of *cueA* transcription by ions of copper, silver, gold and mercury was demonstrated. A non-polar *cueA* deletion mutant (SBW25 *ΔcueA*) displayed two-fold reduction in its tolerance to copper compared with the wild-type strain. However, there was no alteration in the sensitivity of the mutant strains to these metal ions. The experiment to assess the ecological significance of *cueA* showed that fitness of the mutant CSBW25 *ΔcueA* was not affected in vitro, but was compromised in plant environments (roots of sugar beet and pea). The results revealed an ecologically significant contribution to bacterial fitness in the plant rhizosphere and also suggested a possible accumulation of copper ions on plant surfaces (Zhang and Rainey 2007).

The mechanisms of biocontrol activities of bacterial species may vary as in the case of fungi exhibiting biocontrol potential. Antibiosis and competition for nutrients may be exhibited frequently together by the antagonists producing metabolites toxic to pathogens simultaneously. Different strains of the BCA may produce different antibiotics (De La Fuente et al. 2004) *Pseudomonas fluorescens* (*Pf*) 2-79 inhibits *Gauemannomyces graminis* var. *tritici* (*Ggt*) by producing phenazine-1-caboxylate (Thomashow et al. 1990), whereas *Pf* Q2-87 produces 2,4-diacetyl phloroglucinol (DAPG) effective against the same pathogen (Vincent et al. 1991). The antibiotic DAPG (Phl) has antifungal, antibacterial, antiviral, antihelminthic and phototoxic properties. The bacterial strains were found to be effective against several microbial plant pathogens. The fluorescent pseudomonads, in addition to their utility as biocontrol agents may be able to promote the growth of plants colonized by them, providing additional benefit to the treated plants. The bacteria *Bacillus* spp. L324-92, due to its ability to form endospores and to produce antibiotics with a wide spectrum of activity have been shown to be effective against three important diseases caused by *Ggt* (take-all), *R. solani* (root rot) and *Pythium irregulare*

(Pythium root rot) (Kim et al. 1997). *Pf* and *P. putida* act on plant pathogens by chelation of iron compounds (Kloepper et al. 1980; Bagnasco et al. 1998).

The polyketide compound DAPG has been shown to have a wide spectrum of antimicrobial activity. The determinative role of DAPG production in the suppression of a variety of soilborne disease by fluorescent pseudomonads has been indicated by studies on DAPG-deficient mutants (Raaijmakers et al. 1999; Notz et al. 2001). The DAPG-biosynthetic locus in *Pf* strains Q2-87, CHA0, and F113 has been identified. This locus consists of the DAPG-biosynthetic genes *phlABCD* which are flanked upstream by the divergently transcribed *phlE* gene, encoding a transcriptional regulator and downstream by the *phlE* gene coding for putative export protein (Bangera and Thomashow 1999; Delany et al. 2000; Schnider-Keel et al. 2000). The TetR-like regulatory protein PhlF represses the expression of the DPAG-biosynthetic operon by binding to the *phlA* promoter. DAPG itself may act as the depressing signal by dissociating the repressor Phlf from the *phlA* promoter (Haas et al. 2000; Schnider-Keel et al. 2000; Abbas et al. 2002). Another additional regulator protein PhlH encoded by *phlH* gene located downstream of *phlF* has been shown to be a positive regulator of DAPG synthesis in strain CHA0 (Schnider-Keel et al. 2000; Haas and Keel 2003).

The effect of combination of *P. fluorescens* strains CHA0 and Q2-87 which depend on the production of DAPG for the biocontrol activity on the suppression of root diseases of wheat was assessed. DAPG acted as a signaling compound inducing the expression of its own biosynthetic genes. In order to determine the ability of DAPG secreted by one strain to influence expression of DAPG-biosynthetic gene of another strain in vitro and on roots of wheat, CHA0 and Q2-87 strains were combined. DAPG production was monitored by observing the expression of *lacZ* fused to the biosynthetic gene *phlA* of the respective strain. Dual culture assays in which two strains were grown in liquid medium physically separated by a membrane, revealed that Q2-87, but not its DAPG-negative mutant (Q2-87::Tn5-1) strongly induced *phlA* expression in a *ΔphlA* mutant strain of CHA0. Likewise, *phlA* expression in a Q2-87 background was induced by DAPG produced by CHA0. The *phlA* expression was enhanced by DAPG produced, when both strains were together. The results demonstrated that two unrelated pseudomonas could stimulate each other for the expression of an antimicrobial compound required for biocontrol activity (Maurhofer et al. 2004). *P. fluorescens* UP61 produced three antibiotics DAPG, pyrrolnitrin and pyoluteorin involved in its biocontrol activity against *Sclerotium rolfsii* in beans and *R. solani* in tomato. Molecular techniques such as 16S rDNA RFLP, RAPD and rep-PCR and partial sequence of the *phlD* gene revealed the similarity of UP61 with other biocontrol strains isolated from different geographical locations that produce these antibiotics (Maurhofer et al. 2004).

A PCR-based screening method using primers Phl2a and Phl2b was applied to screen strains of *P. fluorescens* (*Pf*) to assess their ability to produce DAPG which was implicated in the biocontrol activity against *Xanthomonas oryzae* pv. *oryzae (Xoo)* causing the bacterial blight disease of rice. Among the 278 strains of *Pf*, a subpopulation of 27 strains was found to be capable of producing DAPG, as revealed by the PCR assay. A 745-bp fragment characteristic of DAPG was am-

plified by the primers employed from the DNA of these strains. DAPG produced by the *Pf* strains inhibited the growth of *Xoo* in vitro and suppressed the disease symptoms (up to 59–64%) under greenhouse and field conditions. The *Tn5* mutants defective in DPAG biosynthesis were less efficient in disease suppression (Velusamy and Gnanamanickam 2003; Velusamy et al. 2006).

Pseudomonas fluorescens (*Pf)* strain KD capable of protecting cucumber plants against *Pythium ultimum* contains the type III secretion system (TTSS) genes considered to originate from the bacterial pathogen *P. syringae*. The role of TTSS in the protection offered by *Pf* was examined. Inactivation of the TTSS gene *hrcV* following insertion of an omega cassette significantly reduced the biocontrol activity of *Pf* against *P. ultimum* on cucumber. However, the mutant was able to colonize roots of cucumber as the wild-type strain. Expression of the operon containing *hrcV* in the strain KD was strongly stimulated in vitro and in situ by the pathogen, but not in planta. The level of activity of pectinase, polygalacturonase (a key pathogenicity factor) from the fungal pathogen was more strongly reduced by the wild-type strain, compared with the mutant strain. The results suggested that the virulence genes acquired horizontally from bacterial pathogens might be functionally recycled in saprophytic *Pseudomonoas* spp. in which the biocontrol potential may be enhanced (Rezzonico et al. 2004).

Biosynthesis and regulation of DAPG production in *P. fluorescens* is controlled by many genes. One of them the polyketide synthase gene *phlD* is required for the synthesis of the DAPG precursor monoacetylphloroglucinol (Bangera and Thomashow 1999). This gene is conserved among DAPG-producing *Pseudomonas* strains. In order to identify strains with high rhizosphere competence and potentiality for biological control of crop diseases, the sequence heterogeneity of *phlD* was assessed. The genotypic diversity of DAPG-producing *Pseudomonas* strains may be determined by amplified rDNA restriction analysis, RAPD analysis and BOX-PCR assay which require isolation and cultivation of *phlD*$^+$ strains from soil and rhizosphere environments. In a later investigation, a simple and rapid method to detect the presence and genotypic diversity of *phlD*$^+$ *Pseudomonas* strains directly in rhizosphere samples without prior isolation or enrichment on nutrient media was developed. Denaturing gradient gel electrophoresis (DGGE) of 350-bp fragments of *phlD* allowed discrimination between genotypically different *phlD*$^+$ reference strains and indigenous isolates. DGGE could be applied for simultaneous detection of multiple *phlD*$^+$ genotypes present in mixtures in rhizosphere samples. Seven *phlD*$^+$ genotypes were identified using DGGE analyses of 184 indigenous *phlD*$^+$ isolates obtained from rhizospheres of wheat, sugar beet and potato plants. The bioassays showed the differential ability of the genotypic groups with respect to colonization of sugar beet, confirming the biological significance of this PCR-DGGE methodology to detect specific *phlD*$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately 5×10^3 CFU/g of root tissues (Bergsma-Vlami et al. 2005).

Bacteriocins secreted by bacteria are proteinaceous toxins that can kill closely related bacterial competitors in the environment. These toxic metabolites constitute a structurally and functionally diverse group within the antimicrobial compounds. *Pseudomonas* spp. colonizing rhizosphere have been reported to produce

bacteriocins. The bacteriocin LipA produced by *Pseudomonas* spp. strain BW11M1 is a novel type of antibacterial protein and it exhibits homology to mannose-binding lectins mostly found in monocots (Parret et al. 2003). The biocontrol agent *P. fluoresecens* Pf-5 has two *llpA*-like genes designated $llpA1_{pf\text{-}5}$ and $llpA2_{pf\text{-}5}$. Recombinant *Escherichia coli* cells expressing $llpA1_{pf\text{-}5}$ or $llpA2_{pf\text{-}5}$ acquired bacteriocin activity and secreted a 31-kDa protein cross-reacting with $LlpS_{Bw11M1}$ antibodies. Analysis of antimicrobial spectrum showed that $LlpA1_{pf\text{-}5}$ and $LlpA2_{pf\text{-}5}$ were able to inhibit *P. fluorescens* strains. Bacteriocin production by BCAs may contribute to their rhizosphere competence. Thus recombinant biocontrol strains equipped with bacteriocin genes may be able to perform better as plant growth-promoting inocula by out-competing rhizosphere-inhabiting pseudomonads (Parret et al. 2005).

Interaction between two BCAs acting on the pathogen through different mechanisms may be either synergistic or counteractive affecting the effectiveness of biocontrol activity. *P. fluorescens* A506 and *Pantoea agglomerans* strains Eh252 and C9-1 suppress fire blight pathogen *Erwinia amylovora* primarily through competitive exclusion and production of antibiotics respectively. An extracellular protease produced by *Pf* strains inactivated the antibiotics mccEh252 and herbicolin O produced by strains Eh252 and C9-1 of *P. agglomerans*. Derivatives of A506 deficient in extracellular protease production were generated by transposon mutagenesis and the *aprX* gene encoding the protease was cloned and sequenced. Strain A506 inactivated mccEh 252 and herbicolin O in plate bioassays. On the other hand, the *aprX* mutant were insensitive to antibiosis. This finding that strain A506 could inactivate antibiotics produced by *P. agglomerans* provides an insight into ways to improve mixtures of two biocntrol agents for suppression of fire blight disease under field conditions (Anderson et al. 2004).

Root colonization by and ecological fitness of PGPR may be affected by many different traits and environmental factors. Strains of most of the 22 genotypes of DAPG producers are phenotypically very similar, but they differ appreciably in the ability to colonize the rhizospheres of certain crop plants (Raaijmakers and Weller 2001). D-genotype isolates have been found to be aggressive colonists of wheat and pea. The gene *dsbA*, a well-conserved gene in fluorescent pseudomonads encodes a periplasmic disulfide-bond-forming enzyme implicated in the biogenesis of exported proteins and cell surface structures. The role of *dsbA* in *P. fluorescens* Q8r1-96, producer of DAPG, in colonization of the roots of wheat and pea was investigated. A *dsbA* mutant of Q8r1-96 exhibited decreased motility and fluorescence and altered colony morphology. However, it could produce more DAPG and total phloroglucinol-related compounds and was more inhibitory in vitro to *Gauemannomyces graminis* var. *tritici* (*Ggt*) than the parental strain, when introduced separately into natural soil. Q8r1-96 and the *dsbA* mutant did not differ in their ability to colonize the rhizosphere of wheat in the greenhouse. However, when two strains were co-inoculated, the parental strain consistently out-competed the *dsbA* mutant. The results showed that *dsbA* did not contribute to the exceptional rhizosphere competence of Q8r1-96 (Mavrodi et al. 2006a). In another study, the role of three genes, an *sss* recombinase gene, *ptsP* and *orfT* which are important in the interaction of *Pseudomonas* spp. with different plant species was investigated. Gene replacement

mutants of Q8r1-96 strain were generated. The *ptsP* mutant was impaired in wheat root colonization, whereas mutants with mutations in the *sss* recombinase gene and *orfT* were not altered in this trait. However, all three mutants were less competitive than the wild-type strain Q8r1-96 in wheat rhizosphere, when they were introduced by co-inoculation into the soil with wild-type strain (Mavrodi et al. 2006b).

6.3 Improvement of Biocontrol Potential

The biocontrol potential of the bioagents may be improved by making modifications in their genetic makeup. Mutations in the genes of BCAs encoding compounds involved in the suppression of plant pathogens or silencing pathogenicity genes and transformation of BCAs by introducing genes from other organisms have been attempted to improve the efficiency of the BCAs.

6.3.1 Fungal Biocontrol Agents

Inducing variants exhibiting loss of pathogenicity following mutation has been attempted. These mutants may be employed as BCA against the same pathogen species. Mutants of *Colletotrichum gloeosporioides* Cg-14 infecting avocado were generated by insertional mutagenesis by restriction enzyme-mediated integration (REMI) transformation. Among the 14 isolates showing reduced virulence compared with wild-type strain, the mutant strain Cg-M-142, when preinoculated, delayed symptom development by the wild-type strain. Furthermore, this mutant could induce resistance to the wild-type strain and this phenomenon was accompanied by an increase in the levels of preformed antifungal compound, diene, from 760 to 1200 µg/g fresh weight at 9 dai. Another mutant Cg-M-1150 failed to produce appressoria, but it exhibited reduced macerating ability on mesocarp and no symptoms on the pericarp. This mutant neither altered the concentration of diene produced nor delayed the appearance of decay symptoms in fruits. This investigation indicated the possibility of employing mutants with reduced pathogenicity (avirulence) as biocontrol agents against anthracnose disease of avocado (Yakoby et al. 2001).

Trichoderma harzianum (*Th*) has a chitinolytic system consisting of five to seven distinct enzymes depending on the strain. The entire chitinolytic system may be essential for maximum efficiency as BCA. The ability of individual enzyme of the complex, the 42-kDa endochitinase (Ech 42) has been examined in different studies. Ech 42 can hydrolyze *Botrytis cinerea* cell walls in vitro and inhibit spore germination and germtube elongation of other fungi (De La Cruz et al. 1992). The gene *ech42* encoding this endochitinase was strongly induced during interaction with other fungi. Expression of *ech42* was repressed by glucose (García et al. 1994). The role of Ech42 in mycoparasitism of *Th* was studied by generally manipulating the gene *ech42*. Several transgenic *Th* strains carrying multiple copies of *ech42* and the corresponding gene disruptants were generated. Under inducing conditions,

the level of extracellular endochitinase activity of multicopy strains dramatically increased up to 42 folds, compared to the wild-type strain. In contrast, the gene disruptant has lost entire enzymatic activity. However, no major differences in the efficacy of the transformants as BCA against *R. solani* and *Sclerotium rolfsii* could be observed in greenhouse experiments (Carsolio et al. 1999). The chitinases Chit 42 and Chit 33 from *Th* CECT 2413 do not have a chitin-binding domain. By incorporating a cellulose binding domain (CBD) from cellobiohydrolase II of *T. reesei* to these enzymes, hybrid chitinases Chit 33-CBD and Chit 42-CBD with stronger chitin-binding capacity were engineered. The transformants of *Th* overexpressing the native chitinases exhibited higher levels of chitinase-specific activity, resulting in greater inhibition of growth of pathogens such as *B. cinerea, R. solani* and *Phytophthora citrophthora*. The results reveal the importance of endochitinase in the antagonistic activity of *Th* strains and the effectiveness of incorporation of CBD to enhance the biocontrol potential of the microorganism against fungal pathogens (Limón et al. 2004).

Trichoderma spp. are more resistant to the products of glucose oxidase activity than fungal plant pathogens, although the BCA does not possess a glucose oxidase ortholog (Kim et al. 1993; Mach et al. 1999). A transgenic strain SJ3-4 of *T. atroviride* expressing the *Aspergillus niger* glucose oxidase-encoding gene, *goxA,* under a homologous chitinase (nag 1) promoter was generated. The expression of *goxA* occurred in transgenic strain soon after contact with fungal pathogen and the glucose oxidase was secreted. Glucose oxidase-containing culture filtrates inhibited germination of *B. cinerea* spores to a greater extent compared to wild-type strain. The growth of *R. solani* and *P. ultimum* was effectively arrested by strain SJ3-4 in culture plates. Under low inoculum levels of pathogens, no distinct difference could be seen in planta. But beans planted in heavily infested soil and treated with strain SJ3-4 germinated, but not the seeds treated with the wild-type strain. Further, SJ3-4 was more effective in inducing systemic resistance in plants. Bean plants protected by SJ3-4 were highly resistant to leaf infection by *B. cinerea*. The results revealed the biocontrol potential of BCA transformed with heterologous genes driven by pathogen-inducible promoters can be enhanced (Brunner et al. 2005).

6.3.2 Bacterial Biocontrol Agents

Salicylic acid (SA) has been shown to be an endogenous signal molecule and exogenous application of SA induces systemic acquired resistance (SAR) to diseases affecting different crops, including tobacco (Volume 3, Chapter 5). The effect of introduction of the SA biosynthetic genes *pchBA* from *Pseudomonas aeruginosa* PA01 into *P. fluorescens* strains P3 and CHA0 on induction of SAR was investigated. Transformation of strain P3 which did not produce SA, with *pchBA* rendered this strain capable of SA production. In addition, the ability of strain P3 to induce SAR in tobacco against *Tobacco necrosis virus* (TNV) improved significantly. Strain CHA0 was capable of producing SA naturally under conditions of iron limitation.

Transformation of CHA0 with *pchBA* enhanced the level of SA production in vitro and in the rhizosphere of tobacco. However, the ability of CHA0 to induce SAR in tobacco did not show any improvement. In planta, suppression of black root rot caused by *Thielaviopsis basicola* by transformed tobacco lines was not improved significantly (Maurhofer et al. 1998).

6.4 Biocontrol Agent-Plant-Pathogen Interaction

Plants have to exist in association with a vast diversity of microorganisms from the moment they are planted as seeds, seedlings or vegetative propagules. The plant-microbe interaction may be beneficial or harmful. Pathogenic microbes induce diseases. The beneficial microbes may exert a direct symbiotic relationship with plants or may protect the plants by suppressing the pathogens or by inducing resistance in the susceptible plants to reduce the ill effects of infection (Volume 3, Chapter 5).

6.4.1 Plant-Biocontrol Agent Interaction

Plant health depends, in part, on the association with disease suppressive microflora. But little information is available on the role of plant genes in establishing different types of association with biocontrol agents. Identification of such plant host genes may contribute to the understanding of the basis of plant health in natural communities and to the development of new disease management strategies. Advances in genetics and molecular biology have provided tools to have an insight into the mechanisms that underpin the BCA-plant interactions. A genetic mapping population of tomato was used to assess the role of plant host in disease suppression. The efficacy of the BCA, *Bacillus cereus* in suppressing the seed pathogen *Pythium torulosum* was investigated. Significant phenotypic variation was observed among recombinant inbred lines that comprise the mapping population for resistance to *P. torulosum*. Disease suppression by *B. cereus* and growth of *B. cereus* were revealed by genetic analysis. As the QTL for disease suppression by *B. cereus* mapped to the same locations as QTL for other traits, it was suggested that the host effect on biocontrol agent might be mediated by different mechanisms (Smith et al. 1999).

The green fluorescent protein (GFP)-encoding gene (*gfp*) has been used for monitoring the fate and behavior of bacterial and fungal inoculants in situ (Bae and Knudsen 2000). GFP, unlike other biomarkers, does not require any substrate or additional cofactors in order to fluoresce and even a single cell expressing GFP can be easily visualized using epifluorescence microscopy or confocal scanning laser microscopy. In situ interactions between *gfp*-tagged strains of *Trichoderma atroviride* and *Pythium ultimum* or *Rhizoctonia solani* that were grown in cocultures and on cucumber seed surfaces during seed germination were monitored. GFP expression under the control of host-inducible promoters was also studied. Induction of specific genes of *T. atroviride* was monitored visually in cocultures, on plant surfaces and in

soil in the presence of colloidal chitin of *R. solani*. This molecular technique allowed monitoring of the initiation of the BCA gene expression cascade (Lu et al. 2004).

It is well known that root exudates have an active role in the regulation of pathogenic interactions. Several species of *Pseudomonas* and *Bacillus* can stimulate root proliferation or have antagonistic effects on pathogens in the rhizosphere. Few studies have focused on the molecular bases of plant responses to rhizobacteria such as *Pseudomonas* spp. By using suppressive subtractive hybridization, the genes of *Medicago truncatula* induced during root colonization by *P. fluorescens* C7R12 were identified. Ten plant genes coding for proteins associated with a putative signal transduction pathway, showed an early and transient activation during initial interactions between plant-BCA interactions. The similar response of the *M. truncatula* genes to *P. fluorescens* and *Glomus mosseae* (mycorrhiza) indicated the possible operation of common molecular pathways in the perception of the microbial signals by plant roots (Sanchez et al. 2005).

The local and systemic expression of defense-related genes in cucumber seedlings inoculated with *Trichoderma asperellum* T203 and challenged with *Pseudomonas syringae* pv. *lachrymans (Psl)* was analyzed by real-time RT-PCR. The analysis showed that *T. asperellum* modulated the expression of genes involved in the jasmonate/ethylene signaling pathways of induced systemic resistance (ISR) (LOX1, Pal 1 ETR1 and CTR1) in cucumber plants. Challenge by *Psl* of cucumber plants pretreated with BCA, led to higher systemic expression of PR-genes encoding for chitinase I, β-1,3-glucanase and peroxidase relative to the noninoculated, challenged plants. The results indicated that the *T. asperellum* induced a potentiated state in the plant enabling it to become more resistant to subsequent pathogen infection (Shoresh et al. 2005).

6.4.2 Biocontrol Agent-Pathogen-Plant Interaction

The molecular biology of the interactions between the BCAs, microbial pathogens and plant hosts has been studied only in very few pathosystems. Different species of *Pseudomonas* suppress the microbial pathogens through different mechanisms. Induced systemic resistance (ISR) by pseudomonads has been investigated more intensively compared with other bacterial inducers. The most often examined component of mechanisms accounting for ISR is the study of signaling pathway in the plant (Van Loon and Glick 2004). In tomato-*Phytophthora infestans* (late blight disease) pathosystem, elicitation of ISR by *Bacillus pumilus* SE34 on tomato lines with various mutations in signaling pathways was tested. ISR was elicited on *nahG* lines that breakdown endogenous salicylic acid (SA), but not in the ethylene (ET)-insensitive NR/NR line or in the jasmonic acid (JA)-insensitive *df1/df1* line (Yan et al. 2002). On the other hand, ISR elicited by *Pseudomonas* spp. is typically independent of SA and does not result in activation of the *PR1a* gene (Van Loon and Glick 2004). When ISR is elicited by selected strains of *Bacillus* spp., some different pathways seem to be in operation, than when ISR is elicited by *Pseudomonas* spp. The specific

signal transduction pathway progressing during induction of ISR by *Bacillus* spp. depends on the strain, the host plant and pathogen-host plant species combination (Kloepper et al. 2004).

Attempts have been made to have an insight into wide range of changes that occur in the expressomes of the BCA, plant and pathogen by using proteomic and functional genomic analysis (Grinyer et al. 2005). A method to separate the proteome of *T. harzianum* strain 252 and *T. atroviride* strain P1 from that of *R. solani, B. cinerea* and *P. ultimum* and that of bean or tomato plants, while all three components are grown together under controlled conditions was developed. A large variety of protein spots differentially expressed during the two-(BCA-pathogen or BCA-plant) or three-(BCA-plant-pathogen) way interaction were selected from the proteome of each component. The *Trichoderma* expressome was strongly and differentially changed when different host-pathogens combinations were used or while the fungal pathogen was colonizing a plant root. The plant proteome contained more than 100 proteins that were turned on or off by the presence of *Trichoderma* spp. Abundance of proteins involved in disease resistance processes such as PR-proteins and signaling proteins was evident in bean and tomato colonized by *Trichoderma* spp. with and without challenge by the pathogen (Woo et al. 2006).

The studies on the proteome of *Trichoderma* spp. interacting with pathogens and plants have provided information to improve the understanding on how these BCAs search for the pathogen, talk to the plant and protect themselves from the toxic metabolites of the pathogens and other microbes in the rhizosphere. The *avr*-like genes and novel protein functioning as typical avirulence compounds have been detected in *Trichoderma* spp. These compounds are likely to have a role in inducing resistance and recognition of nonpathogenic fungus. In order to withstand the impact of toxins produced by microorganisms, the presence of a variety of ATP-binding cassette (ABC) transporters in *Trichoderma* spp. may be an effective way of tackling the microbes threatening their survival. The hyphae of *Trichoderma* are able to penetrate into the root cortex and colonization is limited to the first few cell layers. The mode of action of *Trichoderma* spp. for providing a stimulus for plant defense mechanism resulting in enhancement of host plant resistance to diseases is yet to be understood clearly (Woo et al. 2006).

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Chapter 7
Molecular Biology of Pathogen Resistance to Chemicals

Abstract Application of chemicals to restrict the incidence and spread of different diseases affecting various crops has been the important and largely visible disease management strategy. With the advent of modern fungicides with single-site of action on target pathogens, emergence of resistant strains, has resulted in restricted use or withdrawal of such fungicides/chemicals, seriously affecting the fungicide market. Biochemical and nucleic acid-based molecular techniques have been shown to yield reliable and reproducible results rapidly, facilitating the planning of effective action schedules. The molecular methods can be used for detection, differentiation and quantification of sensitive and resistant strains/isolates of fungal and bacterial plant pathogens. In addition, the occurrence of exotic strains exhibiting resistance can be efficiently detected and contained by taking appropriate measures. The importance of continuous monitoring the populations of different isolates of pathogens coupled with resistance management activities for effective crop disease management is underscored.

Of all the crop disease management strategies, the effectiveness of chemical application has been more perceptible and it has contributed significantly for reduction of crop losses that might have, otherwise, been incurred by the growers. Nevertheless, the emergence of fungicide-resistant strains in the target pathogen(s) has caused great concern and necessitated consideration of alternative strategies to overcome this obstacle for production of quality agricultural produce. Development of pathogen strains resistant to non-systemic protectant fungicides used prior to 1970s was observed occasionally. But with increased application of systemic fungicides with narrow spectrum of activity, the occurrence of fungicide-resistant strains has become more frequent. As the systemic fungicides act on single site in the sensitive fungal pathogens, the development of resistance by substitution of a single amino acid by suitable changes in the DNA sequence of the pathogen becomes possible, as in the case of *Rhynchosporium secalis* causing barley leaf blotch disease. Mutations affecting amino acids at positions 198 and 200 appeared to result in resistance to carbendazim [methyl benzimidazole-2-yl carbamate (MBC)] (Hollomon and Butters 1994). With significant increases in the use of systemic fungicides on high value crops, often being indiscriminate and excessive in nature, incidence of resistant

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strains of several fungal pathogens was reported from different countries. Detecting and monitoring resistance development is the basic requirement for fungicide resistance management. Conventional bioassays based on mycelial growth and spore germination tests are time-consuming and difficult. On the other hand, molecular techniques have been demonstrated to be rapid and reliable in several pathosystems (Narayanasamy 2002).

7.1 Resistance in Fungal Pathogens to Chemicals

Detection and identification of fungicide resistant fungal pathogens rapidly and precisely will be useful to assess the prevalence of resistant strains on the particular host plant species and in different locations. This information will provide a sound basis for monitoring of strains exhibiting resistance to one or more fungicides.

7.1.1 Identification of Fungicide Resistant Strains

Fungal pathogens exhibit different levels of sensitivity or resistance to various fungicides. A bioassay technique, using Alamar Blue as a growth indicator of fungal growth, was employed to test large numbers of isolates of the fungal pathogen for fungicide sensitivity or resistance. This high throughput assay using microtiter plates was developed for detecting sensitivity of *Septoria tritici* to fungicides. Metabolic activity of *S. tritici* in the presence of a normally inhibitory concentration of fungicide could be assessed rapidly by measuring color or fluorescence released after conversion of the growth indicator substrate Alamar Blue incorporated into the medium. Development of pink or blue color showed growth or no growth respectively, indicating the resistance/sensitivity of the isolates of *S. tritici* (Rothamsted Research 2003–2004). *Colletotrichum* spp., causing anthracnose diseases in fruit crops became less sensitive or resistant to several fungicides such as benzimidazoles, diethofencarb, thiophanate methyl and iminoctadinetriacetate (Davidse and Ishii 1995). A single-point mutation in β-tubulin gene was associated with moderate or high levels of resistance to benzimidazole in several pathogens (Ishii et al. 1998; Ishii 2002; Peres et al. 2004). Benzimidazoles bind to the fungal β-tubulin molecule, disrupting microtubule assembly and interfering with cell division during mitosis. Point mutations at the site of action inhibit this conformational change resulting in microtubule resistance to inhibition by thiabendazole (Davidse and Ishii 1995; Paluh et al. 2004).

High level of resistance to benzimidazoles may be acquired in a single step and appears to be controlled by genes of major effect. Generally resistance alleles may exist at very low frequencies in populations not exposed to the fungicide. Detection of point mutations (one-step change) has been possible by employing PCR with allele-specific oligonucleotides (ASOs). Allele-specific amplification of gene segments, determining resistance or sensitivity may be done using ASOs directly as

PCR primers (Williams et al. 1990). Most of the strains of *Botrytis cinerea* could be precisely identified by this procedure (Hollomon and Butters 1994). Resistance to benzimidazoles in *B. cinerea* is governed by a single major gene. Luck and Gillings (1995) reported that all benzimidazole sensitive isolates (ben^{s}) had the sequence GAG (Glu) at codon 198, whereas resistant isolates (ben^{HR}) had a single base substitution to GCG (Ala) at this position. Resistant isolates were identified using the PCR assay. The genetics of resistance in *Phytophthora infestans* to metalaxyl was investigated. A single locus exhibiting incomplete resistance was considered to control resistance to phenylamides (Shaw and Shattock 1991). Likewise, resistance to metalaxyl in *P. capsici* (Lucas et al. 1990) and *P. megasperma* var. *sojae* (Bhat et al. 1993) was attributed to a single incompletely dominant gene. Molecular markers linked to loci controlling resistance in *P. infestans* were identified by using bulk segregant analysis (BSA). Six random amplified polymorphic DNA (RAPD) markers have been identified (Fabritius et al. 1997; Judelson and Roberts 1999). One dominant gene plus minor genes may be involved in phenylamide resistance in *P. infestans* (Lee et al. 1999).

DNA probe techniques are sensitive and rapid, providing results within 24–48 h. They can be used for monitoring resistance in obligate fungal pathogens or slow-growing organisms that may be difficult to isolate in pure cultures. A rapid procedure for detecting fungicide-tolerant strains formed due to continued application of a fungicide is desirable. Primers designed from conserved region of the fungal β-tubulin gene were used for amplification by PCR assay and sequencing a portion of this gene. In the TBZ-tolerant strains *of Helminthosporium solani* due to a point mutation at codon 198, a change in the amino acid sequence from glutamic acid to alanine or glutamine was observed. By using species-specific PCR primers designed to amplify the desired regions in conjunction with a restriction endonuclease to cause cleavage in sensitive isolates, fungicide-tolerant strains could be rapidly detected, facilitating further action for fungicide resistance management (McKay and Cooke 1997; Errampalli et al. 2001). Isolates of *Venturia nashicola* causing scab disease of Japanese pear exhibiting resistance to benzimidazoles exhibited codon changes at position 198 or 200 in β-tubulin genes, as in the case of several other fungal pathogens showing resistance to benzimidazole fungicides (Ishii 2002).

The sensitivity of *Colletotrichum gloeosporioides* and *C. acutatum* isolates from various fruit crops grown in Japan to three fungicides viz., thiophanate methyl, diethanocarb and iminoctadinetriacetate was assessed. In order to determine the relationship between fungicide sensitivity and molecular phylogeny, 51 isolates from 10 fruit crops, acacia and tea were tested. The internal transcribed spacer (ITS) and 5.8S regions or rDNA of these isolates were examined. Based on in vitro analysis, *C. gloeosporioides* isolates were divided into sensitive, intermediate resistant and resistant to these fungicides. In contrast, *C. acutatum* isolates were comparatively less sensitive. The partial sequence of β-tubulin gene of *C. gloeosporioides* highly resistant to thiophanate methyl had the amino acid substitution of Glu (GAG) with Ala (GCG) at codon 198. In intermediate resistant isolates of *C. gloeosporioides*, Phe (TTC) was substituted with Tyr (TAC) at codon 200. On the other hand, the sequence in *C. acutatum* isolates was GAG or AAG at codon 198 and it was the

same at codon 200 as in *C. gloeosporioides*. It is possible that factors other than the mutation of the β-tubulin gene such as ATP-binding cassette (ABC) transporter systems may have a role in reduced sensitivity of cells of *C. acutatum* to benzimidazoles. The molecular genetic analysis revealed that *C. gloeosporioides* and *C. acutatum* are distinct species based on ITS and 5.8S rDNA regions and the results were confirmed by the species-specific PCR primer reaction. Molecular phylogenetic analyses placed the isolates of *C. gloeosporioides* in the same genetic group, whereas isolates of *C. acutatum* were divided into two genetic groups. Although phylogenetic relationship was not closely related to fungicide sensivity, the isolates of *C. gloeosporioides* showing high resistance to iminoctadinetriacetate were included in the same phylogenetic group (Chung et al. 2006).

The restriction sites were identified by using the sequence data on the β-tubulin gene of *Venturia nashicola*. The base change (GAG to GCG) in codon 198 created a *Tha* I restriction site together with a *Hga* I site. The DNA samples bearing such a mutation after treatment with each enzyme yielded two restriction fragments from PCR amplification. This PCR-RFLP protocol was useful to identify the resistant isolate showing negative cross-resistance to diethofencarb. By using allele-specific PCR (ASPCR) primers based on the sequence difference, it was possible to identify mutants in codon 198 or codon 200. Another nucleic acid-based technique, the single-strand conformation polymorphism (SSCP) analysis was employed for the detection of fungicide resistance in *V. nashicola.* Single-strand DNA having one base-change, after denaturation, can be separated on a gel according to the difference of electrophoresis patterns. The PCR products of the β-tubulin gene fragments are separated using capillary gel electrophoresis and fluorescence detection system. The differences in migration time of the DNA sample will reveal a mutation at either codon 198 or codon 200. The components of the mixture of DNA samples from both highly carbendazim-resistant and-sensitive isolates applied onto a gel, were separated as a result of the different conformation of each DNA strand (Ishii 2002).

The isolates (64) of *C. gloeosporioides* from *Limorum* spp. (a herbaceous ornamental perennial plant) were subjected to arbitrarily primed (AP)-PCR. Selected 12 isolates were tested by ITS-1 sequence analysis. Based on these tests, the benomyl-resistant (46 isolates) and-sensitive (18) isolates were separated into two distinct genotypes. Sequence analyses of the β-tubulin genes, *TUB1* and *TUB2* of five sensitive and five resistant representative isolates revealed that the benomyl-resistant isolates had an alanine substitute, instead of glutamic acid at position 198 in TUB2. The results suggested that the resistant and sensitive genotypes were two independent and separate populations (Maymon et al. 2006).

Penicillium digitatum causes green mold disease of harvested citrus fruits. Thiabendazole (TBZ) is the most commonly used fungicide to combat this disease. Mutations at specific amino acid position codon 198 of *TUB2* gene conferred resistance to *P. digitatum* (Koenraadt et al. 1992). In a later study, isolates of *P. digitatum* were collected from California packinghouses and citrus groves (i) to assess their levels of resistance to TBZ, (ii) to identify sensitive and resistant phenotypes and (iii) to investigate the genetic basis of TBZ resistance. Of the 74 isolates tested, 19 were found to be TBZ-resistant. Genetic fingerprinting using RAPD analysis showed

that the isolates selected for β-tubulin gene sequencing were unique genotypes. A 781-bp region of the β-tubulin gene was sequenced to identify mutations relevant to amino acid codon positions 165, 167, 198 and 200. Nineteen TBZ-resistant isolates displayed a point mutation in the β-tubulin gene sequence corresponding to amino acid codon position 200. Thymine was replaced by adenine (TTC → TAC) which changed the phenylalanine (F) to tyrosine (Y). In contrast, no change in the amino acid sequence could be seen in 49 TBZ-sensitive isolates that were sequenced. All isolates of *P. digitatum* resistant to TBZ collected from geographically diverse samples seemed to be due to the mutation at the same position, resulting in conferment of TBZ resistance (Schmidt et al. 2006).

Development of resistance to dicarboximides in fungal pathogens has been reported. Resistance of *Ustilago maydis* infecting corn (maize) to dicarboximide fungicides (DCFs) was investigated. Laboratory mutants of *U. maydis* resistant to the DCF vinclozolin were isolated and characterized. A mutant with single gene resistance to vinclozolin was identified (Orth et al. 1994). The isolate VR43 was used to clone a gene conferring resistance to vinclozolin. A genomic library was constructed and an 87 kb resistance-conferring fragment was isolated and sequenced. The 1218-bp open reading frame (ORF), when disrupted by deletion, did not confer resistance. This ORF contained the *adr-1* gene, whose product showed high percent homology to Ser (Thr) protein kinases. All of the conserved catalytic residues essential for protein kinase activity were present in the *adr-1* gene product. This report appears to be the first to demonstrate the involvement of a protein kinase in conferment of resistance to a synthetic chemical. The protein kinase in the mutant *U. maydis* VR43 might confer resistance indirectly by circumventing inhibition by fungicide at another site in the cell (Orth et al. 1995).

Isolates of *Botrytis cinerea* exhibiting resistance to dicarboximides were first detected in Northern Europe within a few years of application of these fungicides. Isolates of *B. cinerea* showing resistance to fenhexamid exhibited enhanced sensitivity to phenylpyrrole, fludioxonil and iprodione. Two distinct populations of *B. cinerea*, designated Hyd R1 and non-Hyd R1 were distinguished. Hyd R1 isolates showed natural resistance to hydroxanilide, fenhexamid, whereas non-Hyd R1 population was sensitive. The 3-keto reductase gene *ERG 27* was isolated by PCR assay. The gene product displayed striking homology with mammalian 17-HSD7, indicating a common function between Erg 27p-like protein and 17-HSD7 in sterol biosynthesis. The analysis of genetic polymorphism of gene product revealed 12 amino acid differences between strains of Hyd R1 and non-Hyd R1 types that may account for natural resistance of Hyd R1 to fenhexamid. Furthermore, sequence analysis of Erg27p-like protein showed that Hyd R3 isolated from treated populations of non-Hyd R1 strains exhibiting high resistance to fenhexamid had two mutations that could be used as population markers (Albertini and Leroux 2004).

Mefenoxam, the active isomeric form of metalaxyl has been used commonly for the control *of Phytophthora cactorum* causing crown rot disease in strawberry. Resistance to mefenoxam in *P. cactorum* was observed in South Carolina (Jeffers et al. 2004). The levels of sensitivity of population of *P. cactorum* to mefenoxam were assessed. All 132 isolates of *P. cactorum* tested, were found to be sensitive

to mefenoxam at 1 ppm level. Populations of *P. cactorum* in California apparently were sensitive and exhibited host specificity with relatively minor variation in genomic DNA as revealed by AFLP analysis, although resistance to mefenoxam in *P. cactorum* was reported elsewhere. It was suggested that the genetic variation observed in *P. cactorum* included significant geographical and host origin components which form important factors to be considered for disease management (Bhat et al. 2006).

Strobilurins are natural products with fungicidal properties obtained from the Basidiomycete fungi *Strobilurus tenacellus* and *Oudemansiella mucida*. Azoxystrobin derived from *O. mucida* and another strobilurin fungicide kresoxim-methyl were applied for the control of cucumber powdery mildew (*Podosphaera fusca*= *Sphaerotheca fuliginea*) and downy mildew (*Pseudopernospora cubensis*) diseases (Hollomon 2001). However, failure of control of the diseases resulted in withdrawal of these fungicides from official guidelines. The QoI fungicides (inhibitors of mitochondrial respiration at Qo site of cytochrome *bc1* enzyme complex) were considered to have low risk to the environment due to their rapid degradation in soil. QoI fungicides such as azoxystrobin have been used for the control of a broad range of crop diseases. However, outbreaks of resistance to QoIs have posed a serious threat to the growth of fungicide market in their near future. QoI resistance has been detected in the isolates of over 20 different fungal pathogens in different countries around the world. This list includes *Blumeria graminis, Alternaria solani, Pseudopernonospora cubensis, Plasmopara viticola, Botrytis cinerea, Pyricularia grisea* and *Pythium aphanidermatum* (Ishii 2006). Point mutation(s) in the mtDNA, encoding the fungicide-targeted cytochrome b in pathogen seemed to determine high resistance to strobilurins. Fragments of the fungicide-targeted mitochondrial cytochrome *b* gene were amplified from total pathogen DNA using PCR assay. The sequences of amplicons were analyzed to elucidate the molecular mechanism of resistance. A single-point mutation (GGT to GCT) in the cytochrome *b* gene resulted in substitution of glycine by alanine at position 143 in resistant isolates of *P. cubensis* was attributed to this amino acid substitution. On the other hand, the same mutation was detected only in some resistant strains of *P. fusca*. The results suggested that a mutation at position 143 in target-encoding gene due to substitution of an amino acid might be a major cause for rapid development of high strobilurin resistance in these two cucumber pathogens (Ishii et al. 2001).

The partial nucleotide sequence of cytochrome *b* gene in resistant and sensitive isolates of *P. cubensis* were determined. A single point mutation at codon 143 was detected in resistant isolates. The mutated nucleotide sequence was recognized by restriction enzyme *Ita*I, as demonstrated by directly purifying the total DNA from the leaf discs cut from infected cucumber plants, followed by amplification of a fragment of cytochrome *b* gene by PCR. The enzyme *Ita*I cut the PCR products purified from the resistant isolates, but not the products from sensitive isolates of *P. cubensis*. In another experiment to detect the anticipated low proportion of mutated cytochrome *b* gene in mitochondrial cells following stoppage of QoI fungicide application, a rhodamine-labeled reverse primer PCR assay was performed. Only PCR products which carried the mutated DNA were digested with the enzyme *Ita*I.

The ratio of mutated DNA was determined on a polyacrylamide gel, using fluorescence image analyzer. It was possible to detect the mutated DNA at 1% level (Ishii et al. 2002; Ishii 2005).

The strobilurins such as kresoxim-methyl, azoxystrobin, trifloxystrobin and pyraclostrobin with a single-site mode of action are called Qo inhibitors. They inhibit mitochondrial respiration by binding to the Qo site (the outer quinine oxidizing pocket) of the cytochrome *bc1* enzyme complex (complex III) resulting in blocking of electron transfer in respiration pathway and leading to energy deficiency due to lack of ATP (Barlett et al. 2002). Part of this complex is the cytochrome *b* (*cyt b*) located in the mitochondrial genome. The sequences of the cytochrome *b* gene (*cyt b*) are known for many fungal pathogens. As many as 15 different mutations in *cyt b* leading to resistance have been detected. In most cases, resistance was conferred by a single point mutation in the *cyt b* gene resulting in a change at amino acid position 143 from glycine to alanine (G 143 A) (Gisi et al. 2002). In certain pathogens like *Pythium aphanidermatum* and *Pyricularia grisea*, resistance to QoIs was attributed to a change in phenylalanine to leucine at position F 129L (Gisi et al. 2002; Kim et al. 2003).

In a later study, the possibility of resistance development in *Puccinia* spp. that were exposed to QoIs as frequently as powdery mildews was examined, since the powdery mildews were the earliest to develop resistance to QoIs. The fragment of the cytochrome *b* (*cyt b*) gene responsible for the binding site of QoIs was sequenced in *Puccinia* spp. using DNA and RNA as template for PCR and RT-PCR respectively. Degenerated primers for the *cyt b* gene amplified a 450-bp fragment of *P. recondita* f.sp. *tritici* and this fragment was cloned and sequenced. A Thermal Asymmetric Interlaced (TAIL)-PCR approach was applied to characterize the two hot spot regions which included possible mutations that can confer resistance to QoIs (amino acid residues 120–160 and 250–300). Specific primers for the *cyt b* gene of *Puccinia* spp. – the forward Prcytb2C, Prcytb2D and Prcytb2G and the reverse Prcytb2Br and Prcytb2Dr – were designed to amplify different parts of the gene. The primer pair Prcytb2G/Prcytb2Dr was able to amplify specifically the *cyt b* gene fragment from cDNA of *Puccinia* species encoding for the amino acid residues 4–332. Further specific primers for the *cyt b* gene for *P. graminis* f.sp. *tritici, P. striiformis* f.sp. *tritici, P. coronata* f.sp. *avenae, P. hordei, P. recondita* f.sp. *secalis, P. sorghi* and *P. horiana* were designed. The primers can be a valuable diagnostic tool to easily isolate the *cyt b* gene fragment in which point mutation, if occurs, may be detected to identify strains showing resistance to QoI fungicides (Grasso et al. 2006).

The sterol demethylation-inhibitors (DMIs) used as fungicides may act on the sensitive fungal pathogens by binding to the haem of the sterol 14 α-demethylase, inhibiting C14 demethylation of eubricol, inducing accumulation of precursor sterols and reducing contents of ergosterols and/or other desmethyl sterols, eventually resulting in the disruption of membrane function (Ishii 2002). Fenarimol and other DMI fungicides were applied against *Venturia nashicola* causing sacb disease of Japanese pear. As long time was required to detect resistance of *V. nashicola* using conventional methods, a PCR-based technique was developed. Fragments of the

sterol 14α-demethylase gene were amplified by PCR directly using pathogen DNA isolated from scab-infected pear tissues. However, no variations in deduced amino acid sequences of the sterol 14α-demethylase could be observed in the isolates with differing sensitivities to DMI, indicating mechanisms other than point mutations in the targeted gene may possibly be involved in resistance of *V. nashicola* to DMIs (Ishii 2002).

Blumeriella jaapii causes cherry leaf spot (CLS) disease. DMI fungicides were used by about 96% of the growers as per a survey in Michigan State. The widespread use of DMIs resulted in their reduced efficacy. Determination of sensitivity of the isolates of *B. jaapii* by conventional method in petriplates required at least 2 months to yield results, due to extreme slow growth of the pathogen in culture. Isolates with resistance to DMIs were not inhibited at fungicide rates 3 to > 100 times the concentration required for inhibition of sensitive isolates. A pair of primers that could identify DMI resistant (DMI^R) isolates in a PCR protocol was designed. The primers were based on species-specific sequences upstream of the *CYP51* gene that were implicated in overexpression of this gene as in the case of detection of resistant isolates of *Penicillium digitatum* (Hamamoto et al. 2001). The PCR protocol developed in this study could detect DMI^R isolates of *B. jaapii*, since all isolates tested contained the upstream sequences targeted by the primer pairs. Different levels of resistance of isolates could not be determined by this PCR assay. The resistant isolates made up 99.7% of the population of *B. jaapii*, indicating the seriousness of the problem of resistance of *B. jaapii* to DMIs. The overexpression of *CYP51* was found to be the mechanism of resistance development in *B. japii*, indicating the possibility of overexpression of *CYP51* in DMI^R isolates imposing deleterious effects on the fitness of the DMI^R isolates in orchards without DMI selection pressure. The PCR-based procedure has the potential for wide application for rapid detection and identification of resistant isolates of *B. jaapii* facilitating timely decision-making (Proffer et al. 2006).

Triazoles are included in the DMI fungicide group, because they inhibit the formation of cytochrome P450 sterol 14α-demethylase (450_{14DM}) required for the biosynthesis of ergosterol. Cyproconazole has been applied on wheat for the control of Septoria blotch disease caused by *Mycosphaerella graminicola*. Genetic variability in this pathogen is high and also the baseline sensitivity of the isolates of *M. graminicola* shows wide variation (Stergiopoulos et al. 2003). Five *M. graminicola* populations sampled from unsprayed wheat fields in four continents [Australia, Israel, Oregon(USA) and Switzerland] were assayed for eight RFLP markers and their level of tolerance to cyproconazole. The gene *CYP51* encoding the enzyme eburicol 14α-demethylase was sequenced for all isolates. Unimodal, continuous variations in cyproconazole tolerance were observed among isolates of *M. graminicola* sampled from individual fields, consistent with a polygenic mode of inheritance. The population of the pathogen from Switzerland exhibited the highest level of tolerance to cyproconazole. Analysis of the DNA sequencing data revealed that the pathogen population from Switzerland was dominated by isolates with several point mutations and a 6-bp deletion in *CYP51*. Increased resistance recorded in field isolates was related to these point mutations. Oregon population

of *M. graminicola* sampled from unsprayed resistant host cultivar displayed similar gene diversity in RFLP loci, but higher cyproconazole tolerance and quantitative variation in tolerance than fungal population from the same field sampled from an unsprayed susceptible host cultivar (Zhan et al. 2006).

Several mechanisms may operate in the development of DMI resistance in fungal pathogens. Point mutation in the sterol 14α-demethylase gene was observed in the laboratory mutants of *Penicillium italicum* (De Waard 1996) and field isolates of grapevine powdery mildew pathogen *Uncinula necator*. A single mutation, leading to the substitution of a phenylalanine residue for a tyrosine residue at position 136 was associated with high DMI resistance (Délye et al. 1997). Allele-specific (ASP) PCR yielded an amplified DNA fragment of the expected size from all highly resistant isolates of *U. necator* tested (Délye et al. 1997) (Fig. 7.1). Overexpression of a target is another type of mechanism of development of DMI resistance. In DMI-resistant field strains of *P. digitatum*, constitutive expression of 14α-demethylase, *CYP51*(14DM) gene was dramatically enhanced reaching about 100-folds higher than in wild-type sensitive strains. The stimulation of expression of this gene was linked to a tandem repeat of a transcriptional enhancer in the promoter region of this gene (Hamamoto et al. 2000). The cytochrome P450 sterol 14-demethylase (*CYP51A1*) gene was evaluated for its potential role in the development of resistance in *Venturia inaequalis*, causing apple scab disease to DMI fungicide myclobutanil. The gene *CYP51A1* from one sensitive (S) and two resistant (R) strains were completely sequenced. No difference in the sequences of S and R strains in this gene could be discerned. A single base pair mutation that correlated with DMI resis-

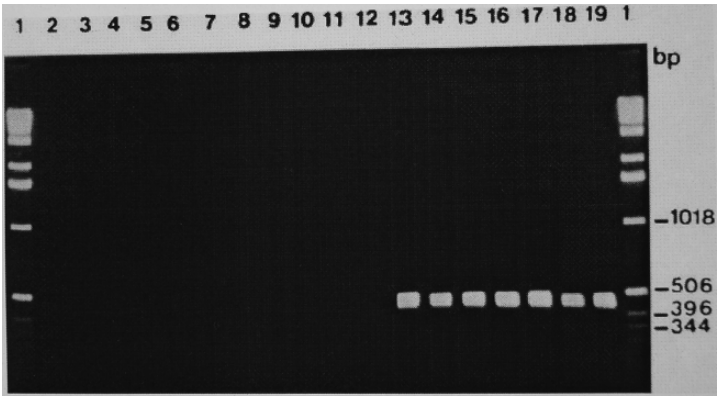

Fig. 7.1 Differentiation of triadimenol-sensitive and – resistant isolates of *Uncinula necator* based on P-450 $_{14DM}$ allele-specific PCR amplification products from genomic DNAs of pathogen isolates.
Lane 1: Molecular weight size markers (standard); Lane 2: Water (negative control); Lane 3: DNA of fungi from grape leaves collected from fields; Lanes 4–8: Isolates sensitive to triadimenol; Lanes 9–11: Isolates moderately resistant to triadimenol; Lanes 12–18: Allele-specific amplification from eight highly resistant isolates. (Courtesy of Délye et al. 1997; American Society of Microbiology, Washington, USA)

tance in strains of *Uncinula necator, Penicillium italicum* and *Blumeria graminis* was also absent in 19 S and 32 R strains of *V. inaequalis* from Michigan, as indicated by allele-specific PCR analysis. Overexpression of *CYP51A1* was significantly greater in 9 of 11 R strains and this enhanced expression was correlated with presence of a 553-bp insertion located upstream of *CYP51A1*. The results suggested that overexpression of the target-site *CYP51A1* is an important mechanism leading to the formation of some field resistant strains of *V. inaequalis*, although other mechanisms may also operate in this destructive fungal pathogen (Schnabel and Jones 2001).

In a later investigation, the mechanism of development of DMI resistance in *V. nashicola* was explored by employing PCR-based cloning techniques that require only nanogram quantities of template DNA for rapidly amplifying target genes. A large fragment of the *CYP51* gene (encoding the target for DMI fungicides, the eubricol 14α-demethylase) from the DMI-sensitive *V. nashicola* strain JS-18 was amplified using degenerate primers. The remainder of the *CYP51* gene was obtained using an adaptor ligation PCR-based cloning kit (Siebert et al. 1995). Sequencing of the CR-2 region of all *V. nashicola* strains did not reveal any difference that can differentiate sensitive and less sensitive strains. In addition, sequencing of the full-length putative *CYP51* protein from the least sensitive fenarimol-sensitive strain ND-8 also did not indicate any change in the predicted amino acid sequence compared with strain JS-18. The results suggested that DMI resistance in *V. nashicola* might be conferred either by enhanced toxicant efflux mediated by the regulation of ABC-transporter homologs or overexpression of the *CYP51* gene itself or the combination of these mechanisms (Cools et al. 2002).

Qualitative resistance to fungicides may be the resultant of mutations in genes encoding fungicide targets. On the other hand, quantitative resistance may be polygenic, affecting the activity of several fungicides with different modes of action. Members of ATP-binding cassette (ABC) transporter family may contribute to fungicide resistance in many fungi (del Sorbo et al. 2000). Many of these proteins can actively transport a wide range of substrates across the plasma membrane (Saurin et al. 1999). The cross-resistance to compounds belonging to the same and different fungicide classes may be due to the broad, overlapping substrate specificity of efflux pumps. The resistance mechanisms in fungal population against fungicides that are applied frequently, have to be understood more clearly to develop effective anti-resistance management strategies. In order to reduce the risk of transporter-based fungicide resistance, it is essential to monitor fungicide sensitivity. Chemical inhibitors that block transporter activity may be important in managing and reducing fungicide resistance problems.

The role of different ABC transporters in fungicide resistance of *Mycosphaerella graminicola*, causing Septoria leaf blotch disease of wheat has been investigated. *M. graminicola* mutants in which individual transporter genes were deleted, lacked clear phenotype. This might be due to distinct but overlapping substrate specificities of the transporters (Zwiers et al. 2003). On the other hand, disruption of the ABC transporter gene *PMPR1* in citrus green mold pathogen *Penicillium digitatum* revealed that this transporter has an important role in determining DMI resistance

(Nakaune et al. 1998). Likewise, the ABC transporter BactrD synergized the MFs transporter Bcmfs1 to mediate DMI resistance in the gray mold pathogen *Botrytis cinerea* (Hayashi et al. 2002).

The isolates of *Pyrenophora tritici-repentis* causing wheat tan spot disease, collected from different fields frequently treated with reduced concentration exhibited reduced sensitivity to strobilurin and azole fungicides (DMIs). Energy-dependent efflux transporter activity can be induced under field conditions and after in vitro application of sublethal amounts of fungicides. A derivative of naturally occurring 4′-hydroxyflavones, 2(4-ethoxy-phenyl)-chromen-4-one, blocks the energy-dependent efflux transporter activity of *P. tritici-repentis*. This inhibitor, when combined with fungicides, rendered the fungicide-resistant strains sensitive to the fungicide, enhancing the effectiveness of chemical control of resistant strains under field conditions. Based on the results of the study, the DMI fungicides expoxyconazole (Opus) and the strobilurin kresoxim-methyl (Discus) remained effective in controlling the field populations of *P. tritici-repentis*, because of the shift in fungicide resistance to sensitivity in the presence of inhibitor of transporter activity. The results bring into focus the role of energy-dependent efflux transporters in fungicide resistance and a novel disease management strategy based on the inhibition of fungicide efflux (Reimann and Deising 2005).

The rice blast pathogen *Magnaporthe grisea* forms melanized appressoria essentially required for penetration of rice plant epidermal cells covered by cuticle layers (Volume 2, Chapter 2). Melanin biosynthesis inhibitors (MBIs) have been widely applied for the control of the rice blast disease. The MBIs are grouped into two classes based on their mode of action: (i) inhibitors of polyhydroxynaphthalene reductase (MBI-R fungicides) and (ii) inhibitors of polyhydroxynaphthalene dehydratase (MBI-D fungicides). There is no report of emergence of resistance to MBI-R fungicides (tricyclazole and pyroquilon) under field conditions. However, in the summer of 2001, disease control efficacy was suddenly reduced following treatment with a MBI-D fungicide carpropamid in Japan (Yamaguchi et al. 2002; Sawada et al. 2004). MBI-D resistance was demonstrated to be due to a point mutation in the gene encoding the targeted protein which led to a single amino acid substitution (V75M) of scytalone dehydratase by using primer-introduced restriction enzyme analysis PCR (PIRA-PCR) (Kaku et al. 2003). Another novel detection system based on hybridization between a fluorescent PCR product and an oligonucleotide probe that could specifically recognize the resistance-type mutation was developed. The PCR-Luminex system distinguished two wild-type and two MBI-D resistant isolates of *M. grisea* effectively based on the signal intensity of hybridization of specific oligonucleotide probes with their corresponding PCR products which carried complimentary nucleotide sequences. This protocol was found to be suitable for a high throughput analysis of single nucleotide polymorphisms (SNPs) (Ishii et al. 2005).

Mandipropamid, a new mandelic acid amide fungicide was found to be highly effective against foliar oomycetes including *Plasmopara viticola*, causing grapevine downy mildew disease. Cross-resistance in field isolates of *P. viticola* among mandipropamid, iprovalicarb, benthiavalicarb and dimethomorph, all members of

the newly defined fungicide class, the carboxylic acid amide (CAA) fungicides and the inheritance of resistance to CAA fungicides in F_1- and F_2-progeny isolates of *P. viticola*, were studied in comparison to that of mefenoxam resistance were studied. A bimodal distribution of sensitivity in field isolates and cross-resistance among all CAAs for a large majority of the isolates could be observed. Crosses between sensitive (s) and CAA-resistant (r) isolates of opposite mating types P1 and P2 were made resulting in the production of oospores abundantly. The parental and five F_1-progeny isolates which were crossed to produce F_2-progeny isolates were characterized with two microsatellite simple sequence repeat (SSR) loci ISA and CES, each containing three loci. Further genotypic characterization of F_2-progeny isolates (32) derived from the three crosses with AFLP markers showed that all were different from each other and from F_1 and F_0 parental isolates in at least 37 out of the 439 polymorphic markers, thus excluding selfing. The results suggested that resistance to CAA fungicides was controlled by two recessive nuclear genes. The risk of resistance in *P.viticola* was determined to be high for phenylamide and moderate for CAA fungicides (Gisi et al. 2007).

7.2 Resistance in Bacterial Pathogens to Chemicals

Development of resistance in bacterial pathogens to antibiotics is known from 1960s. Application of streptomycin resulted in the development of resistant strains of *Erwinia amylovora* in the United States (Schroth et al. 1969). Later, strains of *Pseudomonas syringae* pv. *syringae* (*Pss*) infecting pear resistant to both streptomycin and oxytetracyclines were detected in orchards where both antibiotics were sprayed (Spotts and Cervantes 1995). Resistance of *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) infecting pepper (chilli) and tomato to copper was reported by Bender et al. (1990). The proportion of copper tolerant strains appeared to increase with increase in the frequency of application of copper (Martin et al. 2004). Presence of copper-resistant strains of other plant bacterial pathogens including *Pseudomonas syringae* has been detected (Anderson et al. 1991; Cooksey 1996). Cloning and characterization of both plasmid and chromosomal copper resistance (*cop*) genes indicated that most of the bacterial species were related (Voloudakis et al. 1993; Lee et al. 1994). Expression of resistance to toxic levels of copper appears to involve complex interactions between both plasmid and chromosomal genes. Cloned copper resistance genes from *Xanthomonas* spp. showed homology to the *cop* operon from *P. syringae* (*cop A*) (Voloudakis et al. 1993; Lee et al. 1994).

Further study was taken up to investigate regulation of copper resistance in *X. axonopodis* in comparison to the related *cop* system in *P. syringae*. Copper resistance in strains of *X. axonopodis* pv. *vesicatoria* (*Xav*) was governed by plasmid-borne genes related to the *cop* and *pco* operon of *P. syringae* and *Escherichia coli* respectively. A novel ORF *copL* was needed for copper-inducible expression of the downstream multicopper oxidase product of 122 amino acids rich in histidine and cysteine residues. Transcriptional analysis revealed that *copL* was transcribed con-

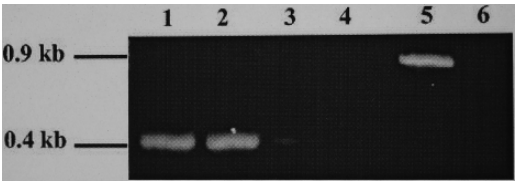

Fig. 7.2 RT-PCR analysis of *copL* and *copA* genes of *Xanthomonas axonopodis* pv. *vesicatoria* 7882. Lanes 1–3: RT-PCR analysis for *copL*; Lanes 4–6: RT-PCR analysis for *copA*. (Courtesy of Voloudakis et al. 2005; American Society of Microbiology, Washington, USA)

stitutively. RT-PCR analysis revealed the presence of a transcript of *copL*, regardless of the presence of copper. The expected size of the amplified product was 396-bp. In contrast, *copA* was expressed only in the presence of copper (Fig. 7.2). No promoter sequence in the intergenic region between *copL* and *copA* was present. The functioning of full copper resistance appeared to be dependent on the presence of an intact *copL* gene. A transcriptional fusion *of Xanthomonas cop* promoter with the *Pseudomonas copABCDRS* was able to confer resistance to copper in *Xanthomonas* showing divergence in the mechanism of regulation of the resistance to copper in plant bacterial pathogens (Voloudakis et al. 2005).

Copper resistance genes in *Xav* were earlier detected on 188 to 200-kb self-transmissible plasmids in strains prevalent in Florida and Oklahoma (Be-nder et al. 1990) and on a 100-kb non-self transmissible plasmid in a strain from California (Cooksey et al. 1990). Later, the presence of chromosome-located genes for copper resistance in *Xav* strain XvP26 occurring in Taiwan was reported (Basim et al. 1999). The similarity of the copper resistance genes in *Xav* and the genes associated with the chromosome of *X. arboricola* pv. *juglandis* (Lee et al. 1994) was investigated. The copper resistance genes in *Xav* strain XvP26 originally isolated from pepper in Taiwan were characterized. Based on pulsed-field gel electrophoresis and Southern hybridization, the copper resistance genes located in a 765-bp region of the chromosome. These hybridized weakly with other copper resistance genes in *Xanthomonas* and *Pseudomonas* strains. Five ORFs in *Xav* generated products with high levels of amino acid sequence identity to the products of copper genes already reported. Copper resistance was abolished by mutations in ORF1, ORF3 and ORF4. On the other hand, an intermediate copper resistance phenotype resulted due to mutations in ORF5, whereas no effect following insertions in ORF2 was observed on copper resistance conferred to a copper sensitive recipient in transconjugant tests. PCR analysis of 51 pepper and 34 tomato copper-resistant *Xav* strains from different regions of Taiwan at different times between 1987 and 2000 and nine copper-resistant strains from United States and South America showed that successful amplification of DNA was obtained only from strain XvP26. The organization of this set of copper resistance genes present in *Xav* seemed to be unique and rare (Basim et al. 2005).

Burkholderia glumae causes rice seedling and seed rot diseases. Rice seeds and rice crops are treated with oxolinic acid (OA) at heading stage for the control of

these diseases in Japan. OA (a quinolone derivative)-resistant strain of *B. glumae* was isolated from rice seedlings grown from OA-treated seeds (Hikichi et al. 1998). OA resistance in *B. glumae* was shown to be due to amino acid substitutions with arginine (Arg) and isoleucine (Ile) at position 83 in GyrA (GyrA83) (Maeda et al. 2004a). The detection of OA-resistant strains rapidly became a necessity to avoid loss due to these strains which could not be controlled by application of OA. A method designated mismatch amplification mutation assay (MAMA) PCR using specific primers base on GyrA83 was developed for detection of *B. glumae* strains resistant to OA in infested rice seeds (Maeda et al. 2004b). In a later investigation, the extent of prevalence of OA resistance in field strains *of B. glumae* isolated from rice seedlings in three prefectures (Mie, Toyama and Iwate) of Japan was investigated. The DNA patterns from rep-PCR and the partial nucleotide sequences of *gyrB* and *rpoD* from different strains were analyzed. The strains (10) from Mie in which GyrA83 was isoleucine (Ile), were divided into two groups based on the band patterns in rep-PCR analysis, although the nucleotide sequences of *gyrB* and *rpoD* were identical among the strains. Two highly OA-resistant Toyama strains were differentiated based on the band patterns in the rep-PCR analysis and the *gyrB* and *rpoD* sequences. These two strains designated Pg-13 and Pg-14 had serine (Ser) and Ile respectively for GyrA83 and were in the same phylogenetic lineage. The results suggested that *B. glumae* might acquire OA resistance faster than phylogenetic diversity as indicated by the repetitive sequences BOX and ERIC and with *gyrB* and *rpoD* sequence determinations (Maeda et al. 2007).

7.3 Fungicide Resistance Monitoring

It is necessary to monitor fungal and bacterial populations for detecting resistance development. In general, a reduction in the fungicide efficacy in controlling the target pathogen is taken as an indication of resistance development. It is very important to detect the early shifts in fungicide sensitivity to resistance in fungal populations. For this purpose, use of bioassay methods has been found to have drawbacks such as long time requirement, labor-intensive nature and inability to identify the genotype (allele) causing resistance. Biochemical and DNA-based methods that provide clear advantages over bioassay methods, are preferred by many researchers. PCR-based techniques have been frequently applied to detect and differentiate sensitive and resistant strains of various fungal pathogens. The rapidity and reliability of results obtained by using appropriate method(s) will facilitate effective disease management by devising suitable resistance management initiatives. Regulation of fungicide use and creating awareness on undesirable effects of improper and excessive application of chemicals have to be taken up, in order to plan for other alternative disease management strategies that can be integrated with reduced use of fungicides. In a recent study, the potential threat of selection for resistance in the populations of *Phytophthora infestans* in the central highlands of Mexico, considered as the putative center of origin, was examined. The fungicides evaluated

were azoxystrobin, cymoxanil, dimethomorph, fluazinam, mancozeb, metalaxyl and propamocarb hydrochloride. The populations of *P. infestans* exhibited a wide range of sensitivities to different fungicides tested. Directional selection toward resistance combined with a reduction in genetic diversity of populations of *P. infestans* was seen only for metalaxyl. The results clearly indicate that continuing vigilance has to be maintained to prevent introduction of exotic strains of *P. infestans* into the country or geographical location desired (Grünwald et al. 2006).

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Glossary

ABC transporters A large family of proteins embedded in plasma membrane characterized by a highly conserved ATP-binding domain; they constitute a class of membrane transporter proteins capable of transferring sugar molecules, inorganic ions, polypeptides, certain anticancer drugs and antibiotics

Acquired resistance Enhancement of resistance of plants following infection or treatment with biotic or abiotic resistance inducers

Actin Structural proteins are present abundantly in eukaryotic cells that can interact with many other proteins; these proteins polymerize to form cytoskeletal filaments

Activator Transcription factor that stimulates transcription

Active site The region of an enzyme that binds substrate molecules and catalyzes an enzymatic reaction

Active transport Export of an ion or small molecule across a membrane against its concentration gradient driven by the hydrolysis of ATP

Alarm signal In response to pathogenic infection, chemical(s) produced, may send signal to host cell proteins/genes leading to synthesis of compounds inhibitory to the invading pathogen

Algorithm A logical description of the manner in which a problem can be solved and that can be used as a specification for how to write a computer program; a computational procedure that employs a combination of simple operations to process, analyze and present pictorially data about sequences of DNA, RNA, proteins or other molecules

Alignment This is an arrangement of two or more molecular sequences one below the other in such a way that regions that are identical or similar are placed immediately below one another

Allele It is an alternate form of a gene occupying a given locus on the same chromosome which controls expression (of product) in different ways

Allozyme An allele of the gene may be involved in the production of an enzyme with properties slightly different from that of the enzyme produced by the gene concerned

Amino acid profile Quantitative delineation of how much of each amino acid is present in a particular protein

Aminoacyl-tRNA synthetase Enzyme that catalyzes the linkage of an amino acid to a specific tRNA to be used for protein synthesis

Amplicon A specific sequence of DNA that is produced from the template fragment by reactions such as polymerase chain reaction assay

Amplification Synthesis of additional copies of a DNA sequence found either as chromosomal or extrachromosomal DNA

Analog A compound or molecule that is a structural derivative of a 'parent' compound; this term also refers to a molecule that may be structurally similar, but not identical to another, and may exhibit many or some of the biological functions of the other

Ankyrin a protein capable of binding spectrin and links the actin cytoskeleton to the plasma membrane

Annealing The process by which the complementary base pairs in the strands of DNA combine

Annotated gene A gene is said to be annotated when it has been recognized from a large segment of genome sequence and if its cellular role is known to some extent

Antibody A protein (immunoglobulin) formed in an animal circulatory system in response to introduction of an antigen (usually a protein or nucleoprotein)

Anticodon The sequence of three bases on tRNA that combines with the complementary triplet codon on mRNA

Antigen A protein or live or inactivated microbe capable of inducing the production of a specific antibody in an animal system and capable of reacting with that antibody in a specific manner

Antisense technology A molecular technique that uses a nucleic acid sequence complementary to mRNA so that the two bind and the mRNA is effectively neutralized

Apoplast Space outside of the plasma membrane of cells constituted by cell walls and conducting cells of the xylem in which aqueous phase of intercellular solutes is present

Apoptosis It is a natural phenomenon encountered in animal cells for the elimination of cells involving a highly regulated, energy-dependent process by which a cell brings about its own death (programmed cell death in plants)

Attacins These proteins constitute a class of antimicrobial peptides produced in the insect hemolymph; synthesis of outer membrane proteins of Gram negative bacteria is inhibited by attacins

Avidin A protein found in egg white and bacteria like *Streptomyces* that binds with very high affinity to the vitamin biotin (vitamin H)

Avirulence Opposite of virulence indicating the inability of a pathogen to infect a particular species or cultivar of a plant, presumably because of the presence of avirulence gene(s) whose product(s) alerts the host resistance mechanism

Avirulence conferring enzyme (ACE) The avirulence gene of the fungal pathogen coding for the enzyme that is recognized by the resistant cultivar carrying corresponding resistance gene. The rice resistance gene *Pi33* recognizes *Magnaporthe grisea* isolates carrying *ACE 1* gene

***Avr* gene** Pathogen gene that is responsible for the inability to infect a given plant species or cultivar

Avr protein The protein coded for by the *avr* gene, capable of functioning as an elicitor of defense responses in plants

Bacterial artificial chromosome (BAC) DNA molecules that are used as cloning vectors, derived from plasmids. This type of vector can be employed for cloning large inserts (of about 150,000 bp) of DNA in bacteria

Base pair (bp) Association of two complementary nucleotides in a DNA or RNA molecule stabilized by hydrogen bonding between their base components adenine pairing with thymine or uracil and guanine pairing with cytosine

Biofilm Bacteria or fungi may be embedded in polysaccharide matrix that plays a role in attachment to host surface, colonization and invasion

Bioinformatics Computer based analysis of data on biological sequencing of the genome of an organism to predict gene function, protein and RNA structure, genome organization and molecular bases in relation to responses of plants to microbial pathogens

Biolistic This term has been coined from the words 'biologic' and 'ballistic'; refers to process involving the use of pellets coated with the desired genes that are fired from a gun into seeds or plant tissues in order to get plants expressing these transgenes

Bioluminescence Emission of light from a living organism

BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) This program is used widely for searching sequence databases for entries that are similar to a specified sequence in question

Bootstrapping A statistics protocol used to estimate the reliability of a result (such as construction of phylogenetic tree) that involves sampling data with replacement from the original set of data

Calmodulin A small cytosolic regulatory protein that binds four Ca^{2+} ions; the Ca^{2+}/calmodulin complex can interact with many proteins resulting in inhibition or activation of those proteins

Capsid Protein coat of viruses that encloses the viral genome

***Cauliflower mosaic virus* 35S promoter (CaMV 35S)** This promoter consisting of DNA sequences of the virus is employed very frequently in genetic engineering to control expression of an inserted gene (synthesis of desired protein in a plant)

cDNA A complementary DNA strand to an RNA strand and synthesized from RNA strand using reverse transcriptase enzyme. It is also known as copy DNA

cDNA clone A DNA molecule synthesized from a mRNA sequence via sequential use of reverse transcription and DNA polymerase

cDNA library A collection of cDNA clones which represent all the genetic information expressed by a given cell or by a given tissue type is known as a cDNA library

Cecropins Antimicrobial proteins isolated from insects capable of forming pores in and causing lysis of bacterial cell membrane (see humoral immunity)

Chaperon protein A protein molecule attached to and to facilitate the export of an effector protein secreted through a type III secretion system of bacteria. It may protect the effector protein from coming in contact with other proteins. The chaperones may also assist with correct folding as the protein molecule emerges from the cell's ribosome

Chemotaxis Capacity of a cell or organism to move toward or away from certain chemicals

Chromosome walking A technique employed for determining the location of and sequencing a given gene within the genome of the organism, by sequencing specific DNA fragments that overlap and span collectively

cis-acting protein This protein has the exceptional property of acting only on the molecule of DNA from which it was expressed

Cistron Viral sequences of nucleotides of DNA/RNA that code for particular protein and are considered to be analogous to genes of other organisms

Clade A clade consists of all the species descending from an internal node of genealogical tree

Clone Genetically identical individuals produced asexually from one individual

Cloning of DNA Introduction of a segment of DNA from one species into the DNA of another species leading to the formation of many copies of the hybrid DNA by replication

Codon A sequence of three nucleotide (triplet) bases on mRNA that interacts with the anticodon on tRNA and specifies the incorporation of a specific amino acid into a polypeptide during translation process

Codon usage The frequency of occurrence of each codon in a gene or genome, especially the relative frequencies with which synonymous codons are used

Colony hybridization A technique used in situ hybridization to identify bacterial colonies carrying inserted DNA that is homologous with some particular sequence (used as probe)

Complementary sequences Two nucleic acid sequences that may form an exactly matching double strand as a result of A-T and G-C pairing. Complementary sequences run in opposite directions

Constitutive genes These genes code for certain protein products that are required at all times for general cell maintenance and metabolism. These genes are expressed as a function of the interaction of RNA polymerase with the promoter without additional regulation. They are also known as household genes

Contigs Overlapping DNA segments that as a collection, form a longer and gapless segment of DNA

Cosmid It is a larger insert cloning vector, useful for analysis of highly complex genomes; forms an important component of many genome mapping projects; cosmid vectors are constructed by incorporating the *cos* sites from the phage λ

Cross-protection Enhancement of resistance in plants following infection by one virus or its strain(s) to reinfection by the same virus or its strains/related viruses

Cytoplasmic resistance Resistance attributed to the genetic material present in the cell cytoplasm

Cytoskeleton A three-dimensional network of fibrous elements comprising primarily of microtubules, actin microfilaments and intermediate filaments found in the cytoplasm of eukaryotic cells; the cytoskeleton offers structural support for the cell and permits directed movement of organelles, chromosomes and even the cell

Cytosol Unstructured aqueous phase of cytoplasm excluding organelles, membranes and insoluble cytoskeletal components

Dalton A mass unit used to indicate the size of a biomolecule; one dalton is equivalent to molecular mass of a hydrogen atom

Data mining A computerized program used to search for relationships between and overall patterns among the data available within a database

Defensins Defense-related, cysteinerich, antimicrobial peptides present in the plasma membrane of host plant species; they constitute a group designated defensins capable of providing resistance against microbial plant pathogens

Denaturation The conformation of a protein or nucleic acid may be drastically altered because of disruption of various bonds due to heating or exposure to chemicals; this may result in loss of biological function(s)

Dendrogram A branching diagram that shows the relative sequence similarity between many different proteins or genes to indicate the phylogenetic relationships; typically horizontal lines indicate the degree of differences in sequences, while vertical lines are used for clarity to separate branches

DNA fingerprinting/ DNA profiling This technique involves the use of restriction enzymes and electrophoresis to determine the differences and similarities in the DNA of individual organisms

DNA library This represents a collection of cloned DNA molecules/fragments of entire genome or DNA copies of all mRNAs produced by a cell type (cDNA library) inserted into suitable cloning vector

DNA ligase The enzyme that seals breaks in DNA strand and also catalyzes the formation of the final phosphoester bond in DNA replication

DNA microarray Oligonucleotides or fragments of cDNAs are printed on a glass slide or membrane filter at a high density, permitting simultaneous analysis of thousands of genes by hybridization of the microarray with fluorescent probes

Domain A portion of polypeptide chain that folds into a compact globular units of the protein forming the basic unit of the tertiary structure and remains distinct even within the tertiary structure of the protein; a discrete part of a protein with its own distinct function

Downstream A relative direction of DNA sequence, as the DNA is usually written with 5' end to the left; downstream would be to the right of a reference point – eg. the start codon is downstream of the protmoter

Ectopic A gene inserted in an unnatural location

Effectors These molecules can manipulate host cell structure and function, thereby facilitating infection (virulence factors/toxins) and /or triggering defense responses (avirulence factors/elicitors)

Effector proteins Bacterial virulence determinants injected into host cells via type III secretion system (TTSS) of the bacterial pathogen

Elongation factor A group of nonribosomal proteins required for continued translation of mRNA (protein synthesis) following initiation; guiding the elongation phase of transcription or translation, during protein synthesis

Endoplasmic reticulum (ER) An extensive network of membrane-enclosed tubules and sacs involved in protein sorting and processing as well as in lipid synthesis

Epitope A specific group of atoms on an antigen molecule that is recognized by the specific antibody produced against the antigen concerned; it is also called as antigenic determinant

Episome An independent genetic element (DNA) that is present inside the bacterial species in addition to the normal bacterial cell genome. The episome can replicate either as an autonomous unit or as one integrated into host genome

Exon A segment of an eukaryotic gene that contains a coding sequence and this part of gene sequence is transcribed into an mRNA and translated to give rise to a specific domain of the protein

Expressed sequence tags (ESTs) These are partial sequences of cDNA clones in an expressed cDNA library; useful for identifying all unique sequences (genes) to determine their functions; a profile of mRNAs allowing cloning of a large number of genes being expressed in a cell population

Expression vector A modified plasmid or virus that carries a gene or cDNA into suitable host cell in which it directs synthesis of the encoded protein; expression vector can be used to screen DNA libraries for a gene of interest or to produce large amounts of a protein from its cloned gene

Expressivity The intensity of realization of the effect of a gene in a phenotype; the degree to which a particular effect is expressed by individuals

Extracellular matrix Secreted proteins and polysaccharides that fill the spaces between cells and bind cells and tissues together

Flagellin These proteins associated with flagella of bacteria, are capable of functioning as a receptor system for general elicitors

Flanking sequence A segment of DNA molecule that either precedes or follows the region of interest on the molecule

Flux The flow of intermediates in metabolism; the rate at which substrates enter and exit a pathway

Footprinting This is a technique employed for identifying protein-binding regions of DNA or RNA based on the ability of a protein bound to a region of DNA or RNA to protect that region from digestion

Free radical A molecule that has one or more unpaired electrons

Functional genomics Functions and interactions of genes or groups of genes belonging to host plants, pathogens or both are genetically examined

Gene cloning Characterization of gene functions by isolating and multiplying individual gene or sequences by insertion into bacteria (*Escherichia coli*); the sequences are duplicated as the bacteria multiplies by fission

Gene expression Conversion of genetic information within a gene into an actual protein or cell process; an overall process applied to assess the information encoded in a gene that can be converted into an observable phenotype (production of a protein)

Gene expression profiling Identification of specific genes that are 'switched on' in a cell at particular point of time or process, enabling the precise definition of the phenotypic condition of that cell

Gene flow Movement of genes (under examination) through specific process, from one population to another population geographically separated apart

Gene knockouts Genetically modified individuals containing either a defective gene or lacking a gene

Gene silencing Interrupting or suppressing the activity of desired gene(s), resulting in the loss of coordination for production of specific proteins

Gene targeting Insertion of antisense DNA molecules into selected cells of the organism in order to block the activity of undesirable genes such as oncogenes

Genetic code The correspondence between nucleotide triplets and amino acids in proteins; the sequence of bases in mRNA specify the amino acid sequence of a polypeptide, read in triplets (codons), based on a set of rules

Genome sequencing Reading of all nucleotides present in the entire genomic DNA of the organism in an orderly way

Genomic library Contains a collection of recombinant DNA clones that collectively constitute the genome of the organism (see DNA library)

G protein A family of membrane–bound cell-signaling proteins regulated by guanine nucleotide binding

Green fluorescent protein (GFP) A protein from jellyfish possessing the property of fluorescing; useful as a marker in fluorescence microscopy

GUS gene Production of β- glucuronidase (GUS protein) in certain organisms such as *Escherichia coli* is controlled by this gene; it is commonly used as a marker gene for genetically engineered plants

Haplotype A collection of alleles in an individual that are located on one chromosome; alleles within a haplotype are, often, inherited as a single unit from one generation to another; it also refers to a group of genomic variations found repeatedly in many individuals within a population

Harpins Proteins coded by *hrp* genes present in the type III secretion system (TTSS) of phytobacterial pathogens; these proteins can induce resistance in susceptible plants

Heterotrimeric G protein A guanine nucleotide-binding consisting of three subunits

High-throughput methods Large number of genes or gene products can be studied using these partially automated protocols

Homologs The individuals considered to have sequences that are evolutionarily related by descent from a common ancestor; proteins or genes exhibiting similarity (homology)

Homology A sequence of amino acid in two or more proteins that are identical to each other; nucleic acid homology indicates the presence of complementary strands that can hybridize with each other

Horizontal gene transfer Incorporation of a 'foreign' gene acquired from an unrelated species into the genome of another organism

Host-specific toxins (HSTs) Some pathogens elaborate toxins that can induce all primary symptoms as the pathogen itself; production of HSTs is governed by specific genes which are expressed in susceptible plants

Hot spots Events such as mutations with unusual high frequency may occur in certain sites in genes, termed as hot spots

Humoral immune response In response to infection, rapid production and secretion of soluble blood serum components occurs in the animal body

Humoral immunity By injecting viable nonpathogenic or inactivated phytopathogenic bacteria into insects, formation of new proteins in the hemolymph of these insects is induced leading to the development of humoral immunity; these proteins have antibacterial properties; cecropins and attacins are formed in the hemolymph of *Hyalophora cecropia*

Hybridization probes DNA or RNA molecules that are complementary to a region in DNA; useful for detecting specific gene(s) in fingerprinting

Hybridoma A hybrid cell line produced by fusing a myeloma (capable of multiplying indefinitely) with a lymphocyte (capable of producing antibodies); the hybridoma provides continuous supply of specific immunoglobulins (antibodies)

Hypersensitive response (HR) A protective or defense response of plants to infection or inoculation with pathogen in which the initially infected cells and the adjacent ones express the phenomenon of programmed cell death (PCD) resulting in self destruction to cordon off the infected area and to restrict the spread of pathogen to other cells/tissues (see programmed cell death)

Idiotype The specific site of antibody molecule capable of combining with the specific site in the antigen (epitope) is said to have an idiotype (for that epitope), serving as an identifying characteristic of an epitope

Indel An insertion or deletion occurring in a protein or nucleic acid sequence; frequently it may be difficult to find out whether a change in a sequence is due to a deletion in that sequence or an insertion of a related sequence

Indexing Testing the plants or seeds or propagative plant materials for the presence of microbial pathogens by biological and/ molecular techniques

Induced systemic resistance (ISR) Colonization of roots of plants by plant growth promoting rhizobacteria (PGPRs) induces systemic resistance to pathogens infecting tissues/organs far away from the roots of plants; this type of resistance to disease is referred to as induced systemic resistance

Inducible promoter The promoter in which start or increase of promotion occurs usually in the presence of a pathogen or toxin or toxic metabolites of the pathogen leading to initiation of defense-related activity

in silico Experimental process performed on a computer and not by bench research

Intergenic sequence DNA sequence between two genes; sometimes called as 'junk DNA'

Intron The sequence for protein synthesis is absent in the intron; this noncoding sequence of the gene interrupting exons is cut out of the mRNA (splicing) prior to translation

Isoforms Different forms of the same protein whose amino acid sequences differ slightly and whose general activities are similar; isoforms may be encoded by different genes or by a single gene whose primary transcript undergoes alternative splicing

Isozymes Different forms of an enzyme involved in the same or similar reactions, but need different optima for their activity

Karyopherin A family of nuclear transport proteins that function as an importin, exportin or occasionally both; each karyopherin binds to a specific signal sequence in a protein (cargoprotein) to be imported or exported

Killer toxin (KT) A proteinaceous toxin isolated from microorganisms like yeast has antimicrobial properties

Knockout gene Selective inactivation of a desired gene by replacing it with a non-functional (disrupted) allele in an otherwise normal organism

Lectins Plant proteins capable of binding to specific carbohydrates; they can be used to detect the carbohydrates in situ

Leucine-rich repeats (LRR) Segments of amino acids containing multiple copies of leucine present repeatedly in a protein; these proteins are known as LRR proteins

Ligand A molecule other than an enzyme substrate that binds tightly and specifically to a macromolecule, usually a protein forming a macromolecule-ligand complex

Linkage On the same chromosome two different loci governing two different traits may be inherited together; closer the loci greater are the chances of linkage

Linker A short segment of ds-DNA that can be ligated onto a second fragment of DNA to facilitate the cloning of that fragment. Linkers contain a restriction site so that they can be ligated to produce the desired sticky ends for ligation

Locus The locatable position of a gene on a chromosome; all alleles of a particular gene occupy the same locus

Marker-assisted selection (MAS) A known resistance gene or chromosomal sequence closely linked to a gene that is used as a genetic marker to select the progenies of crosses or genotypes containing the selected marker by DNA testing; this procedure is very useful in hastening the development of cultivars with built-in resistance to crop diseases

Messenger RNA (mRNA) An RNA molecule serving as a template for protein synthesis

Metabolome It represents the entire metabolic content of a cell or organism; the complete set/complement of all metabolites and other molecules involved in or produced during a cell's metabolism

Microarray A glass slide or silicon chip onto which several DNA probes are deposited for simultaneous determination of gene expression levels of many genes in the same tissue sample

MicroRNA (miRNA) A naturally occurring short non-coding RNA that can act to regulate gene expression

MicRNAs (messenger-RNA-interfering complementary RNAs) These complementary RNA molecules bind to the RNA transcripts of specific genes resulting in blockage of their translation; they are also called antisense RNAs

Molecular cloning Insertion of a desired DNA fragment into a DNA molecule (vector) that can replicate independently in a host cell

Molecular markers DNA sequence (s) that is characteristic of the DNA segment/gene is used for comparing or detecting the similarities of related organisms or genotypes (see marker assisted selection)

Monocistronic Messenger RNAs encoding a single polypeptide chain

Monoclonal antibody An antibody secreted by a clonal line of B lymphocytes

Monophyletic A group of species on a phylogenetic tree sharing a common ancestor that is not shared by species outside the group; a clade is a monophyletic group

Motif A sequence of amino acids or nucleotides that perform a particular role and is often conserved in other species or molecules

Movement proteins Virus coded-specific proteins involved in the movement of plant viruses in the host plants

Mutagen A chemical capable of inducing a high frequency of mutations

Nonhost resistance Resistance exhibited by a plant species on which the pathogen in question is unable to establish infection; the plant species is termed as a nonhost and the interaction is called as incompatible

Ontogenic resistance The level of resistance of plants may vary with the growth stages of host plant species; plants are highly susceptible to viruses in the early growth stages (seedling), but they develop resistance progressively as the plants become older

Open reading frame (ORF) Region of a gene which contains a series of triplet coding for amino acids without any termination codon is known as ORF; the sequences contained in the ORF may be potentially translatable into a protein

Operon One or more genes may be present in this gene unit; they specify a polypeptide and an operator regulates the structural gene

Orthologs Sequences from different organisms (species) that are evolutionarily related by descent from a common ancestral sequence and later diverged from one another as a result of speciation

Pathogenicity factors The factors (genes) of pathogen origin, are essential for initiation of infection and further colonization of plants

Pathogenesis-related (PR) proteins PR proteins are coded for by the host plant, but induced only in pathological or related conditions. They are produced postinfectionally during plant-pathogen interactions; they have different roles in the development of resistance to the diseases caused by pathogens; these proteins are classified into 14 families; PR genes/PR proteins are widely used as marker genes/ proteins to study the defense mechanisms of plants

Peptide mapping Following partial hydrolysis of a protein, a characteristic two dimensional pattern may be recognized (on paper or agar gel) due to the separation of a mixture of peptides

Phytoalexins These are low-molecular antimicrobial compounds that accumulate at the site of infection by incompatible pathogens. Several biosynthetic enzymes are involved in the production of phytoalexins and hence highly coordinated signal transduction events are required

Phytoanticipins Toxic compounds naturally present in the plants that can affect the development of pathogens adversely

Plasmid Represents an independent, stable, self-replicating piece of extrachromosomal DNA in bacterial cells; it does form a component of normal cell genome, but does not get integrated into the host chromosome

Point mutation A mutation resulting in a change in only one nucleotide in a DNA molecule

Polycistronic Coding region representing more than one gene may be present in the mRNA; it codes for two or more polypeptide chains; RNA genomes of plant viruses (such as *Tobacco mosaic virus*) are polycistronic

Posttranscriptional modification A set of reactions that occur to change the structure of newly synthesized polypeptides

Primer A short nucleic acid sequence containing free 3'-hydroxyl group that forms base pair with a complementary template strand and functions as the starting point for addition of nucleotides to copy the template strand

Probe Defined RNA or DNA fragment radioactively or chemically labeled that is used to detect specific nucleic acid sequences by hybridization

Programmed cell death (PCD) Death of cells at the site of initiation of infection by the pathogen as the early response of the host; these cells have a predetermined function amounting to suicide to prevent further spread of the pathogen to other cells/tisssues (see hypersensitive response)

Promoter A region of DNA upstream of a gene that can act as a binding site for a transcription factor and ensure the transcription of the gene concerned

Proteasome A large protease complex that degrades proteins tagged by ubiquitin

Proteome The entire range of proteins expressed in a specified cell

Proteomics Comprehensive analysis of all cell proteins

Pseudogene It is the nonfunctional copy of the gene

Quorum sensing The ability of bacterial or fungal propagules to sense the concentration of certain signal molecules in their environment prior to activation of infection process

Reactive oxygen species (ROS) Intermediate and radical species generated from oxygen such as superoxide or hydrogen peroxide as resistance response in plants

Recognition factors Specific receptor molecules or structures on the host plant surface useful for recognizing the presence of a pathogen

Recombinant DNA A hybrid DNA formed by incorporation of DNA segment from one species into another species

Recombinant protein A polypeptide synthesized by transcription of the hybrid DNA and translation of the mRNA

Regulatory proteins These proteins can bind to DNA and influence the action of RNA polymerase thereby acquire the ability to control the rate of protein synthesis

Reporter gene It is a gene inserted into the DNA of a cell capable of reporting the occurrence of signal transduction or gene expression

Repression of gene function Inhibition of transcription or translation by binding of a product of a gene (repressor protein) to a specific site in the DNA or RNA molecule

Restriction endonucleases Hydrolytic enzymes capable of catalyzing the cleavage of phosphoester bonds at specific nucleotide sequences in DNA

Restriction site This is a specific nucleotide segment of (base pairs) in a DNA molecule that can be recognized and cleaved by the restriction endonuclease enzyme employed

Reverse genetics A method of analysis of gene function by introducing mutations into a cloned gene

Reverse transcription Synthesis of DNA from an RNA template using appropriate reverse transcriptase

RNA-induced silencing complex (RISC) Large multiprotein complex associated with a short ss-RNA that mediates degradation or translational repression of a complementary or near complementary mRNA

RNA interference (RNAi) Degradation of mRNAs by short complementary double-stranded RNA molecules

RNA processing Refers to various modifications that are made in RNAs within the nucleus

RNA splicing A process that results in removal of introns and joining exons in pre-mRNA

Sequencing The process used to determine the sequential arrangement of amino acids/nucleotides in protein /nucleic acid molecule

Serotypes Groups of an organism differentiated based on the variations in the serological reactions with different antibodies; monoclonal antibodies are frequently used for characterization of serotypes of plant pathogens

Signaling Communication established between and within cells of an organism

Signal molecules Host factors (molecules) that respond to the presence or initiation of infection by a pathogen and transmit the signal to and activate proteins or genes in the tissues away from the site of pathogen entry leading to restriction of pathogen spread/disease development

Signal transduction Reception, conversion and transmission of 'chemical message' by a cell

Single nucleotide polymorphism (SNP) Variation detected in individual nucleotides within a DNA molecule; SNPs usually occur in the same genomic location in different individuals

siRNAs Specific short sequences of dsRNA of less than 30-bp in length that can trigger degradation of mRNA containing the same sequence (present in siRNA) within the cell as part of process known as RNA interference (RNAi)

Site-directed mutagenesis A laboratory protocol employed to modify the amino acid sequence of a protein

Somaclonal variation Variability detected in different calli for various characteristics, including disease resistance; the calli exhibiting resistance to disease(s) may be regenerated into whole plants that are tested for the level of resistance to disease(s)

Sticky ends Exposed complementary single strands of DNA can bind (stick) to complementary single strands in another DNA molecule, producing a hybrid piece of DNA

Synteny Two genetic loci are presumed to be linked to the same chromosome, whether or not linkage has been demonstrated, as against the asyntenic loci that are linked to different chromosomes

Systemic acquired resistance (SAR) Resistance to diseases caused by microbial pathogens can be induced by biotic and abiotic inducers of resistance in treated plants; a set of genes referred to as 'SAR genes' is activated by the inducers resulting in the systemic resistance in various organs/parts of plants

Tat pathway The twin-arginine translocation (Tat) system is involved in physiological functions of bacterial pathogens; the Tat pathway operates in the inner membrane of Gram negative bacteria

TATA box A conserved sequence in the promoter of several eukaryotic protein-coding genes where the tranascription initiation complex assembles

Transactivating protein A protein that 'switches on' a cascade of genes /gene regulation

Transactivation Activation of transcription by binding a transcription factor to the DNA regulatory sequence in question

Transcripts During transcription of a gene, various segments of mRNA, known as transcripts, are formed

Transcription The genetic information contained in one strand of DNA is used as a template and transcribed through the enzyme-catalyzed process to specify and produce a complementary mRNA strand; the genetic information in the DNA is rewritten into mRNA

Transcription factor A protein that binds to a regulatory region of DNA, often upstream of the coding region and influences the rate of gene transcription

Transcriptome The entire set of all gene transcripts (mRNA segments derived from transcription process) in a specified organism; provides the knowledge of their roles in the growth, structure, health and disease of the organism concerned

Transduction Bacterial genes may be transferred from one bacterium to another by means of bacterial virus (bacteriophage)

Transformation This a process by which free DNA may be transferred directly into a competent recipient cell; plant cells may be transformed with genes from different kinds of organism to enhance the level of resistance to microbial pathogen(s)

Transgenic An organism carrying in its genome one or more DNA sequences (transgene) from another organism

Translation Synthesis of a polypeptide chain from an mRNA template

Transposon A DNA sequence (segment or molecule) capable of replicating and inserting one copy (of itself) at a new location in the genome

Trypsin inhibitor (TI) protein This protein is constitutively expressed in mature maize kernels of resistant maize genotype at higher levels compared to susceptible leaves

Tubulin A family of globular cytoskeletal proteins that polymerize to form microtubules

Two-hybrid system The yeast or bacterial system that is employed for detecting specific protein- protein interaction; the protein of interest is used as a 'bait' to 'fishout' proteins that may bind to it (referred to as 'prey')

Ubiquitin A small protein present in all eukaryotic cells that has an important role in 'tagging' other protein molecules; the tagged protein molecules are said to be 'ubiquitinated'

Upstream A relative direction of nucleic acids often used to describe the location of a protmoter relative to the start transcription site; the start codon is upstream of the stop codon

Vector An agent used to carry foreign DNA in recombinant DNA technology; natural carrier of microbial pathogens, commonly viruses from infected plant to healthy plant resulting in the spread of the pathogen/disease under natural conditions

Virulence factors Genes or factors essential for and contribute to the virulence of the pathogen; may not be required for the growth and reproduction of the pathogen

Yeast two-hybrid system An experimental technique of detecting the protein-protein interactions in yeast cells

Zinc finger domain A kind of DNA- binding domain consisting of loops containing cysteine and histidine residues that bind zinc ions

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