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Global Trends in Crop Protection

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Bhumi Publishing

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PREFACE

Similar to how veterinarians and doctors treat animals and people, plant doctors and pathologists treat plants. Both biotic (caused by fungi, bacteria, viruses, nematodes, and parasitic plants) and abiotic (caused by temperature, chemical toxicity, mineral deficiency, etc.) plant diseases are identified, and the management of these diseases through cultural practises, biological and chemical control, and host resistance are studied. Being a good plant doctor or pathologist requires having a solid foundation in all aspects of plant pathology. The present book entitled "Global Trends in Crop Protection" covers various area of plant pathology viz. Novel strategies for plant disease management, Molecular basis of plant pathogen interaction, Mechanism of induced systemic resistance (ISR) in plants, Resistance genes (R-genes) in plant defense mechanism, Role of pathogenesis related proteins (PRPs) in plant disease management, Role of phytoalexins in plant defense mechanism, Genetic engineering for plant disease resistance, Exploitation of chitinase genes in plant disease resistance, Serological techniques for detection of virus and Role of molecular techniques in nematology.

The purpose of the book is to make plant pathology easier to understand and to help readers appreciate the subject matter through concepts based on the curricula of several agricultural universities as well as other universities where plant pathology is offered as a minor course. I genuinely believe that this book will help beginners understand the fundamentals of plant pathology quickly and effectively. The constructive criticism and insightful suggestions from readers, especially students and instructors, about how to enhance the book are always greatly appreciated and will be incorporated into later editions.

ABOUT THE BOOK

The goal of the book is to introduce students to the intellectual excitement and difficulties that are currently present in the study of plant disease and its control. The infectious pathogens (fungi, bacteria, fastidious vascular-colonizing bacteria, viruses, sub-viral pathogens, nematodes) and the non-infectious, abiotic agents that induce illnesses owing to mineral imbalances, ozone, PAN, and ethylene each have their own dedicated chapters. The discussion of the chemical factors that determine pathogenicity and virulence, including the cell wall-degrading enzymes, plant hormones, and toxins, is supported by examples from the literature. Without the aid of antibodies, T-cells, and other immune system components, disease resistance mechanisms are just as complex as the mammalian immune system. These include the already present and those that are brought about by molecular interactions between the elicitors and resistance proteins, the products of the avirulence and resistance genes, respectively. Signal molecules play in the emergence of acquired systemic resistance, such as salicylic acid, jasmonates, and ethylene. Induced systemic resistance and wound-induced systemic resistance are thoroughly examined to support their current state as cutting-edge plant pathology concepts. The well-known "gene for gene hypothesis" and the phenomena of specificity and recognition are clearly explained. The physiology and biochemistry of the infected plant are altered, particularly in terms of membrane damage, ionic imbalance, respiration, photosynthesis, protein, and phenol metabolisms. The efforts made by humans to safeguard crops through the use of fungicides and, more recently, biological control techniques such as transgenic resistant cultivars, are explained.

ACKNOWLEDGEMENT

We wish to express my deep sense of gratitude and indebtedness to those all who helped me directly and indirectly during the preparation of this edition of book. We are extremely grateful to our seniors, colleagues, students and friends whose constant inspiration made the work successful. The present work is an outcome of the dedicated efforts of the co-editor student group and faculty members of Hemvati Nandan Bahuguna Garhwal University (Central Garhwal University), CPGSAS (CAU), OUAT and RCA, MPUAT. We cannot forget the whole hearted cooperation received from our family members during the course of writing this book. We gratefully acknowledge the cooperation of creative team of BHUMI PUBLISHING and Dr. S. A. Vhanalakar.

We sincerely hoping the information compiled in the book will serve as a knowledge pool for the researchers, students and extension workers extensively. However, inspite of our best efforts, there may be something in the book requires more attention in the further edition. Any constructive criticism from the readers is always welcome.

- Authors

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CONTENT

Chapter 1 NOVEL STRATEGIES FOR PLANT DISEASE MANAGEMENT

Shaik Munnysha, Ramavath Abhi, Nitisha Gahlot and Manisha

Abstract:

It is well known that plants can protect themselves from pathogen invasion through a range of local, constitutive, or inducible defence mechanisms. A physiological "state of improved defensive capability" known as "induced resistance" is one in which a plant's natural defences are strengthened in response to subsequent biotic threats. Against a wide variety of diseases and parasites, this improved state of resistance is effective. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two types of induced resistance that are most precisely defined, and they can be distinguished based on the type of elicitor and the regulatory mechanisms involved. When induced Arabidopsis plants are inoculated with a pathogen, leaves expressing SAR show primed expression of SA-responsive defense-related genes but not JA/ethylene-responsive genes, whereas leaves expressing ISR are primed to produce JA/ethylene- but not SA-responsive genes.

Some rhizobacteria produce salicylic acid (SA) at the root surface, which activates the salicylic acid (SA) dependent SAR pathway. In other instances, rhizobacteria activate an ISR signalling pathway rather than SA. In *Arabidopsis thaliana*, the existence of an ISR route that is not dependent on SA has been shown. In contrast to pathogen-induced SAR, ISR caused by *Pseudomonas fluorescens*WCS417r needs plant hormone response to ethylene and jasmonic acid (JA), not SA accumulation or pathogenesis-related (PR) gene activation. Mutant investigations revealed that ISR adheres to a new signalling pathway in which elements of the ethylene and JA responses are sequentially engaged to start a defensive state that, like SAR, is regulated by the regulatory factor NPR1. It's interesting to note that an increased level of protection is obtained by simultaneously activating the JA/ethylene-dependent ISR pathway and the SA-dependent SAR pathway. In order to optimise disease control, it is therefore attractive to combine both types of induced resistance.

Keywords: Induced resistance, disease control, plant-pathogen interactions, SAR, ISR, salicylic acid, Jasmonic Acid, Ethylene.

Introduction:

Currently, the use of fungicides, bactericides, and insecticides-chemical substances harmful to plant invaders, causal agents, or vectors of plant diseases-is the main method of disease control. However, the quest for novel, risk-free methods of disease control is strongly required by the hazardous impact of these compounds or their degradation products on the environment and human health. Since the late 1950s, a growing body of data has been gathered

on the phenomena of induced resistance, which has successfully been used in practice during the past ten years. It is well known that plants can protect themselves from pathogen invasion through a range of local, constitutive, or inducible defence mechanisms.

What is induced resistance?

Induced resistance is a physiological "state of enhanced defensive capability" that is triggered by particular environmental cues and increases a plant's natural defences against upcoming biotic threats. Against a wide variety of diseases and parasites, this improved state of resistance is effective. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two types of induced resistance that are most precisely defined, and they can be distinguished based on the type of elicitor and the regulatory mechanisms involved.

The term "induced resistance" refers to the increased expression of a plant's innate defensive mechanisms against various diseases that is triggered by external influences of various kinds and becomes apparent after a subsequent inoculation.

A plant's ability to defend itself against a wide range of infections and pests is said to be enhanced by induced resistance, which develops following the right kind of stimulation. Acquired resistance (SAR) and "induced resistance" (IR) are used interchangeably.

Inoculating plant roots with non-pathogenic rhizobacteria results in the development of resistance in the leaves, which is referred to as "induced systemic resistance (ISR)". The leaves of Arabidopsis plants that had been treated with the nonpathogenic, root-colonizing bacteria Pseudomonas fluorescens displayed this novel form of induced resistance to the bacterial leaf pathogen *Pseudomonas syringae.*

Plant pathologists believe two distinct processes are responsible for warning the plant of pathogen infection, which is misleading concerning plant defence. Systemic acquired resistance and induced systemic resistance are the two terms most frequently employed.

The exposure of the plant to pathogenic, avirulent, and nonpathogenic microorganisms can cause SAR. A specific amount of time is needed for the creation of SAR, during which pathogenesis-related proteins (chitinase and glucanase) and salicylic acid are accumulated depending on the plant and elicitors. Researchers have discovered that diseases that require a living host, such as viruses, rust fungus, and powdery mildew fungi, are most affected by this pathway.

Plant growth-promoting rhizobacteria (PGPR), of which strains from the genus Pseudomonas that do not obviously harm to the plant's root system are the best characterised, enhance ISR. ISR, in contrast to SAR, relies on pathways controlled by jasmonate and ethylene rather than accumulating pathogenesis-related proteins or salicylic acid. Necrotrophic pathogens appear to be more resistant to this mechanism.

Historical background:

Ray and Beauvene (1901), using *Botrytis cinerea*, were the first to recognise the natural phenomena of resistance development in response to pathogen infection (gray mold). Ross (1961) conducted the first controlled laboratory study on SAR and found that inoculating a single tobacco leaf with the tobacco mosaic virus (TMV) lessened the severity of subsequent infections on other plants. For the resistance that appeared in the untreated areas of plants that had been inoculated with TMV, he came up with the term "SAR."

ISR vs. SAR:

- Induced systemic resistance is induced by non-pathogenic rhizobacteria.
- Systemic acquired resistance is induced systemically after inoculation with necrotizing pathogens, HR, or application of some chemicals (SA analogs or agonists).
- Induced systemic resistance is independent of salicylic acid, but involves jasmonic acid and ethylene signaling.
- Systemic acquired resistance requires salicylic acid as signaling molecule in plants.
- Induced systemic resistance is accompanied by the expression of sets of genes distinct from the PR genes.
- Systemic acquired resistance is accompanied by induction of pathogenesis related proteins.
- Both call for NPR1

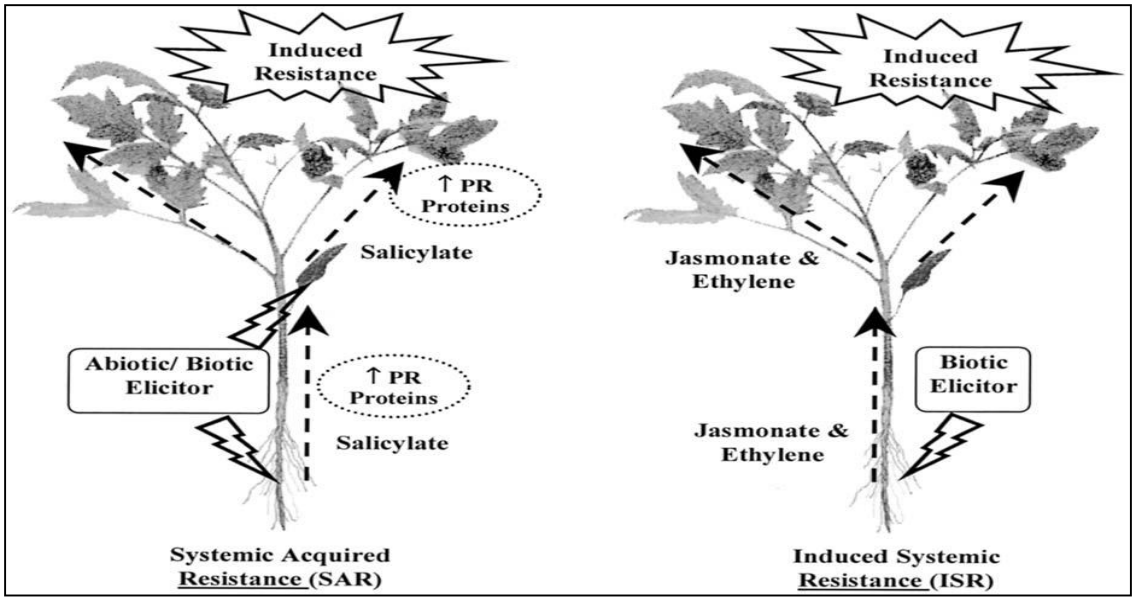


Fig. 1: A pictorial comparison of the two best characterized forms of induced resistance in plants

Both which lead to similar phenotypic responses. Systemic acquired resistance is dependent on the phytohormone salicylate (salicylic acid), and it is linked to the buildup of pathogenesis-related (PR) proteins when root or foliar tissues are exposed to abiotic or biotic

elicitors. Induced systemic resistance is not correlated with the buildup of PR proteins and is dependent on the phytohormones ethylene and jasmonate (jasmonic acid), independent of salicylate, when roots are exposed to specific strains of plant growth-promoting rhizobacteria (or transcripts). However, as evidenced by their dependence on a functioning form of the gene NPR1 in *Arabidopsis thaliana*, both responses are interwoven molecularly.

Cross talk between Salicylic Acid–Jasmonic Acid

Numerous interactions between hormonal signaling pathways have been found in studies conducted over the past ten years. Generally, SA inhibits JA signaling through mechanisms that are (partially) reliant on NPR1, SSI2, WRKY transcription factors, and MPK4, however there has been evidence of synergy between the two signaling pathways. Diverse pathogens have different infection methods that SA and JA regulate; SA signaling mostly fights off biotrophic pathogens and viruses, whereas JA signaling guards against necrotrophic pathogens and insects. Plants appear to balance the costs and advantages of several defence responses because one pathway represses the other. Although there is a bidirectional antagonistic relationship between SA and JA, the primary flow of regulation appears to be the inhibition of JA signaling by SA-dependent stimuli. Different SA and JA signaling mutants were used in microarray investigations, and the results showed that much more JA-dependent genes were suppressed by JA signaling than SA-dependent genes. Additionally, competing studies using diseases and insects that are both biotrophic and necrotrophic showed that SA was given priority over the JA pathway in Arabidopsis. The outcome of the resultant cross talk can be influenced by environmental variables, plant pathogen types, and concentrations of SA and JA in relation to one another. For instance, pre-infection of Arabidopsis with virulent *P. syringae* reduced JA-marker gene expression and resistance to the necrotrophic pathogen *Alternaria brassicicola*, but not pre-infection with avirulent strains of the same pathogen, which cause a quicker, stronger SA response accompanied by an HR. Changes in the cellular redox status appear to be at least partially responsible for controlling SA-JA cross talk. An inhibitor of glutathione production eliminated SA's antagonistic effects on JA signaling. Additionally, Arabidopsis's glutaredoxin 480 (GRX480) suppresses the expression of PDF1.2. In vivo, GRX480 interacts with TGA factors and is expressed by SA in an NPR1-dependent way. GRX480 overexpression, however, slightly decreased SA's ability to induce PR-1. It essentially stopped MeJA's induction of PDF1.2 in a TGA2/5/6-dependent, NPR1-independent way.

Induced-resistance systems in plants

Induced resistance in plants is a tremendously complex system that has only been partially understood in a number of model plant species, including *Arabidopsis*. In *Arabidopsis*, there are three widely acknowledged routes for induced resistance.

Two of these are involved in the direct production of pathogenesis-related (PR) proteins. In one of these pathways, the production of PR proteins typically results from an attack by pathogenic microorganisms, whereas in the other, PR proteins typically result from injury or plant pathogens that cause necrosis. Both of these pathways, however, have different mechanisms for induction. The wounding pathway typically uses jasmonic acid (JA) as the signaling molecule, whereas the pathogen-induced pathway typically uses salicylic acid (SA), which is produced by the plant. When administered exogenously, these substances and their counterparts produce comparable effects, suggesting that there is likely significant cross-talk between the pathways. Induced systemic resistance (ISR), the name given to the JA-induced pathway, is also used to describe several distinct processes that are started by rhizobacteria. The synthesis of a cascade of PR proteins, including antifungals like chitinases, glucanases, and thaumatins, as well as oxidative enzymes like peroxidases, polyphenol oxidases, and lipoxygenases, distinguishes the salicylate- and jasmonate-induced pathways, respectively. Phytoalexins, low-molecular weight molecules with antibacterial capabilities, can also build up. The third form of induced resistance is known as rhizobacteria induced systemic resistance (RISR), which resulted in the establishment of systemic resistance to plant diseases. It is caused by non-pathogenic root-associated bacteria. The PR proteins and phytoalexins are not produced by rhizobacteria colonising roots when plant-pathogenic microbes are not present, therefore it is functionally very different. When a plant is attacked by a pathogen, its defences are stronger and the disease is less severe. In the absence of the protein cascade that is typical of the SA-induced system, RISR produces the possibility for plant defence responses.

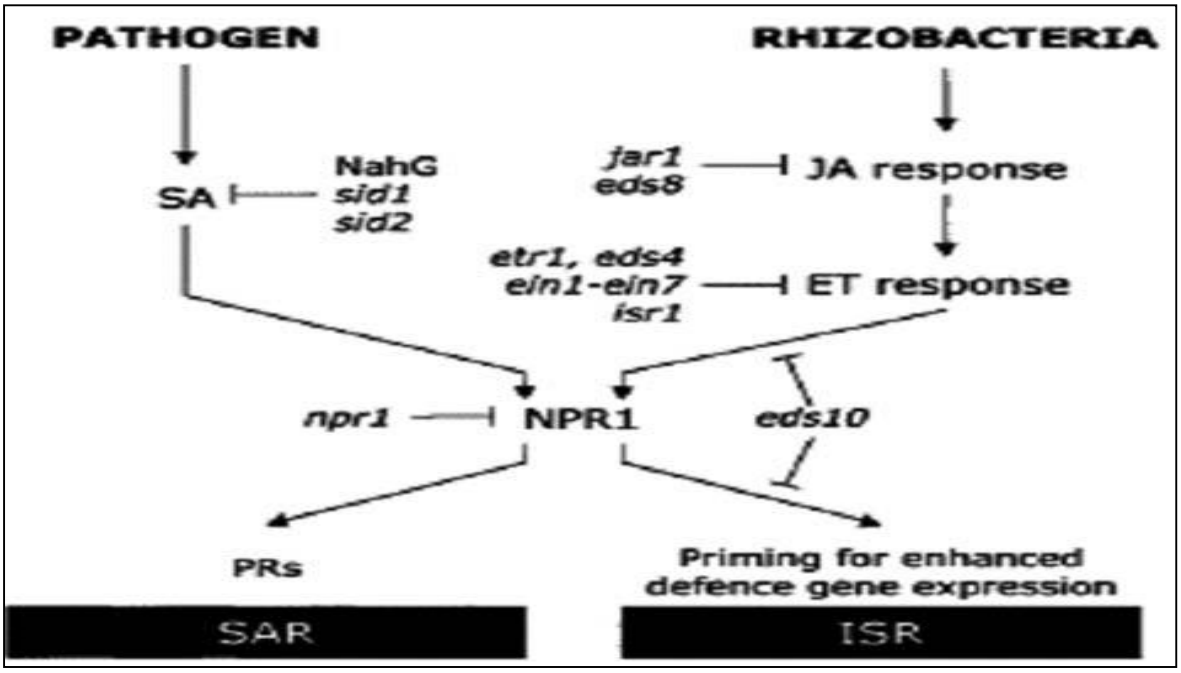


Fig. 2: Schematic model describing the pathogen induced SAR and the Rhizobacteria mediated ISR signal transduction pathway

Systemic Acquired Resistance:

SAR signal transduction pathway

A vast number of genes, including those that encode PR proteins, are concurrently upregulated during SAR, which is accompanied by a local and systemic increase in the endogenous level of SA. Many PR proteins have antibacterial properties and are hypothesised to contribute to the condition of resistance. When SA or an analogue of SA (BTH, INA) is applied exogenously, SAR is induced and PR proteins are activated. On the other hand, transgenic NahG plants that express the NahG gene from the bacterial salicylate hydroxylase are unable to accumulate SA and are compromised in SAR. Mutant and transgenic plants have been used to study the identification of crucial steps in the signal transduction pathway for SAR. In the realm of genetics, Arabidopsis has distinguished itself as a superior model species. A number of mutants were discovered during genetic searches for SAR-compromised Arabidopsis mutants, all of which appeared to be impacted by the same gene. Non-expressor of PR genes (npr-1) or nim-1 were the names given to this gene (non – inducible immunity). After a pathogen infection, mutant mpr-1 plants accumulate normal levels of SA but are unable to express PR genes, which suggests that NPR-1 functions downstream of SA in the SAR pathway. Ankyrin-like repeats, which are known to mediate protein-protein interaction and protein with various functions, are found in the protein that the NPR-1 gene encodes. NPR-1 is physically engaging with a subclass of basic leucine zipper protein transcription factor that bind to promoter regions necessary for SA inducible PR gene expression when SAR is induced. This interaction activates PR genes expression in the nucleus. Under the influence of SA, a redox shift induces NPR1 oligomers in the cytoplasm to dissociate into monomers. These monomers are carried into the nucleus, where they interact with certain TGA transcription factors to enable the production of genes for proteins involved in disease (PRs). These findings gave rise to the theory that the existence of PRs is necessary for the status of SAR.

Accumulation of SA is required for SAR signal transduction

A significant amount of data points to the importance of SA in SAR signaling and disease resistance. Initially, it was discovered that after pathogen infection, the level of SA increased several hundred times in tobacco or cucumber, and it was demonstrated that this rise correlated with SAR. These facts raised the possibility that SA was involved in SAR signaling, together with the discovery that exogenous SA can promote SAR and SAR gene expression. Analysis of transgenic plants expressing the bacterial nahG gene, which codes for salicylate hydroxylase, an enzyme that catalyses the conversion of SA to catechol, provides compelling evidence in favour of this hypothesis. These plants are unable to both accumulate free SA and establish a SAR defence against bacterial, fungal, or viral pathogens, demonstrating that SA accumulation is necessary for SAR induction.

Persistence of SAR

Many illnesses and decay processes in woody tissue take years to develop, however it is unknown whether triggering a SAR response may slow down an infection of an existing disease in a tree. A sub-lethal attack one year later might actually strengthen rather than diminish a tree's resistance, according to the intriguing concept. This is because the SAR response in spruce *(Picea abies*) has been observed to continue for at least one year and potentially longer following inoculation. SAR can be used to generate long-lasting, all-encompassing disease control. A few days after being inoculated with *Pseudomonas lachrymans* or *Colletotrichum lagenarium*, cucumbers became resistant to 13 different diseases, including those brought on by fungi, bacteria, and viruses. Cucumber plants were protected from a single SAR-inducing infection for 44 weeks, whereas broad-spectrum season-long resistance was produced by a booster inoculation two to three weeks after the first infection.

SAR induced by biological agents

Although there are several commercially available organisms that can be used as bio control agents, almost all of them are based on the direct antibiotic concept.

An extract of *Rheynoutria sachalinensis*, according to Kessmann *et al*. (1994), effectively controlled powdery mildews and other crop diseases. The infiltration of *Penicillum janczewskii* or its culture filtrate into melon and cotton increased peroxidase activity (a response associated with SAR), which led to increased protection against *Rhizoctonia solani* and the elimination of the incidence of dampening-off symptoms. Extracts from Bacillus subtilis have been reported to induce resistance in barley, particularly against powdery mildew (Steiner *et al*. 1988).

SAR induced by chemicals

Chemicals that can induce SAR would have a lot of advantages over the present standard methods for treating tree diseases. The SAR reaction would spread across the canopy even if only a section of it was sprayed, offering long-term resistance. Similarly, SAR maintains its effectiveness regardless of unfavourable environmental conditions like low temperatures, in contrast to the traditional biological control approach based on a predator-prey relationship. Three requirements must be met, according to Kessmann *et al*. (1994), for a chemical agent to be categorised as a "activator" of the SAR response:

1) The treated plants are resistant to the same number and type of diseases as those plants in which SAR has been biologically induced.
2) The chemical used has no direct antimicrobial activity or can be converted by the tree into antimicrobial metabolites, and
3) The same pre-infectional biochemical processes are induced as recorded in plant tissues after biological induction of SAR**.**

Modes of action of SA

However, it has been suggested that H_2O_2 functions as a second messenger of SA in SAR signaling as the mechanism by which SA produces SAR is uncertain. Catalase is a protein that binds SA; SA was discovered to decrease the catalase action of this protein, resulting in increased amounts of H_2O_2. Additionally, it was discovered that H_2O_2 induced the expression of the PR-1 gene and was hypothesised to produce SAR. Clearly show that H_2O_2 is not SA's secondary messenger in the SAR signaling process. H_2O_2 levels should rise in uninfected leaves of tobacco plants during SAR activation for H_2O_2 to operate as a signaling agent of SA. This was examined by injecting TMV into tobacco leaves and observing the development of H_2O_2, PR-1 mRNA, and the establishment of SAR. SAR gene expression and SAR establishment did not correspond with an increase in H_2O_2 levels in the uninfected leaves of inoculated plants. Additionally, H_2O_2 was directly examined by infiltrating tobacco to see if it could induce PR-1 expression. After 1 M H_2O_2 infiltration, which also resulted in significant tissue injury, significant PR-1 mRNA accumulation was seen. However, 1 M H_2O_2 did not significantly increase PR-1 in NahG plants, showing the need for SA in H_2O_2-mediated PR-1 production. In fact, it was discovered that high H_2O_2 concentrations induced SA synthesis in both tobacco and Arabidopsis, indicating that H_2O_2 might cause PR-1 accumulation via triggering SA synthesis. Together, our findings show that H_2O_2 is not a secondary SA messenger in the signal chain that establishes SAR. What is the biological importance of H_2O_2's indirect involvement in SAR signaling, and how does SAR's suppression of catalase affect that process? This finding may not have much significance for plant-pathogen interactions, for example. In vitro activity of several heme-iron-containing enzymes, such as catalase, ascorbate peroxidase, and the mitochondrial enzyme aconitase, is reportedly inhibited by very high levels of SA, according to recent research, potentially as a result of SA's purported ability to chelate iron. It's possible that the effect of SA on enzyme inhibition isn't really significant biologically. As an alternative, lesion formation might be greatly aided by the inhibition of catalase and peroxidase. According to reports, the *Kd* for SA's interactions with catalase and ascorbate peroxidase are 14 pM and 78 pM, respectively. This level of SA is present right next to a pathogen-induced lesion, but not in uninfected leaves, where SA levels are 10 to 100 times lower. Therefore, local responses in infected tissue may be the extent of the biological importance of SA-mediated inhibition of oxidoreductases. The improved resistance of cucumber cotyledons to the funga1 pathogen *Colletotrichum lagenarium* was connected with this conditioning of cells by SA, which was dependent on protein synthesis. In addition, the rise in H_20_2 concentrations was not brought on by a slower rate of degradation but rather by a rise in H_20_2 synthesis. The information now available thus points to the possibility of multiple SA modes of action in resistance reactions. A high-affinity SA receptor may be involved in the stimulation of SAR gene expression in uninfected leaves. In contrast to tissues

where SAR has not yet been developed, tissues with established SAR are capable of rapidly inducing an oxidative burst at the site of pathogen infection. High SA concentrations at the infection site in infected leaves may inhibit catalase and other oxidoreductases. Catalase activity inhibition might increase the oxidative burst and extend the half-life of H_20_2. A number of local defence mechanisms, such as programmed cell death during the HR, the activation of defence genes, and the manufacture of SA in neighbouring cells, may be activated by the oxidative burst. As a result, a runaway cycle would develop, resulting in high concentrations of SA and H_20_2 near the pathogen assault site.

SAR genes

A group of genes known as SAR genes, some of which encode pathogenesis-related (PR) proteins, are expressed when SAR is established in tobacco and *Arabidopsis*. It has been determined that some PR proteins are acidic β-1,3-glucanases (BGL2) and chitinases (PR-3), which may have the ability to hydrolyze microbial cell wall constituents. As a result, the buildup of PR proteins has been frequently suggested as the underlying molecular cause of SAR. For instance, plant transformation and the cloning of PR genes have yet to produce a single instance in which an inducible acidic glucanase or chitinase, either individually or collectively, increases resistance to fungal infections. As a result, it seems that PR proteins have little effect on SAR.

Mechanisms of SAR

The expression of SAR is governed by a series of molecular and metabolic actions. It begins with the recognition of inducers (pathogens, chemicals), which leads to the production of signal molecules that are transported over vast distances and the activation of numerous mechanisms that help plants develop their defensive capability upon secondary inoculation. Binding of pathogen-derived compounds (elicitors) or chemical substances with receptor sites on plant membranes or cell walls results in the perception of inducers. In-depth study is being done on the generation and nature of signals, as well as the method of their translocation and interactions. Salicylic acid is frequently acknowledged as a signal molecule or a necessary component for the creation of signals in SAR; jasmonate and ethylene are implicated in signalling upon resistance expression brought on by rhizobacteria (ISR). Both SAR-mediating signal routes may work concurrently, producing an additive impact, and enter MAP kinase-based signal-transduction cascades. The expression of the so-called SAR-genes is then triggered by interaction with gene promoters or other regulatory factors. This family of nine genes, whose expression is linked to the start of SAR, is referred to as the "SAR-genes" as a whole. The SAR-genes for tobacco infected with TMV code for the proteins PR-1, glucanase (PR-2), chitinase (PR-3), hevein-like protein (PR-4), thaumatin-like and osmotin-like protein (PR-5), PR-1 (basic), basic class III chitinase, acidic class III chitinase, and PR-Q'. The roles that PR-proteins often play in SAR may be connected to this engagement. As a result, several PR-proteins (glucanase,

chitinase) have hydrolytic activity, implying a lytic effect on pathogen cell walls composed of glucans or chitins. The interaction of members of the PR-5 protein family (thaumatin-like and osmotin-like) with membrane elements results in conformational changes, the evaporation of the pH membrane gradient, and the creation of membrane holes. Lipoxygenase, hydroxyproline-rich glycoproteins (HRGP), and callose systemic induction in non-inoculated leaves may suggest a significant function for fatty acid derivatives and structural components associated to cell walls in SAR. The systemically induced peroxidase is crucial for the cross-linking and reinforcement of cell walls, with the latter serving as a sign of the induced condition. It is suggested that oxidative burst regulates SAR expression. It may be assumed that the deployment of SAR related events allows the plant to respond more rapidly and effectively to a subsequent, "challenge" inoculation.

It is also crucial to remember that the use of SAR prevents the emergence of new disease strains that can outwit the defences of the induced plants. This could be explained by the fact that, as was already said, different components with distinct functions are engaged in SAR, meaning that pathogens have access to a variety of molecular targets. In contrast to what is seen when fungicides or transgenic resistant plants are applied, this circumstance does not exist. In these situations, there aren't many obstacles to overcome, which encourages the emergence of novel plant-pathogenic strains (Lyon *et al*., 1995).

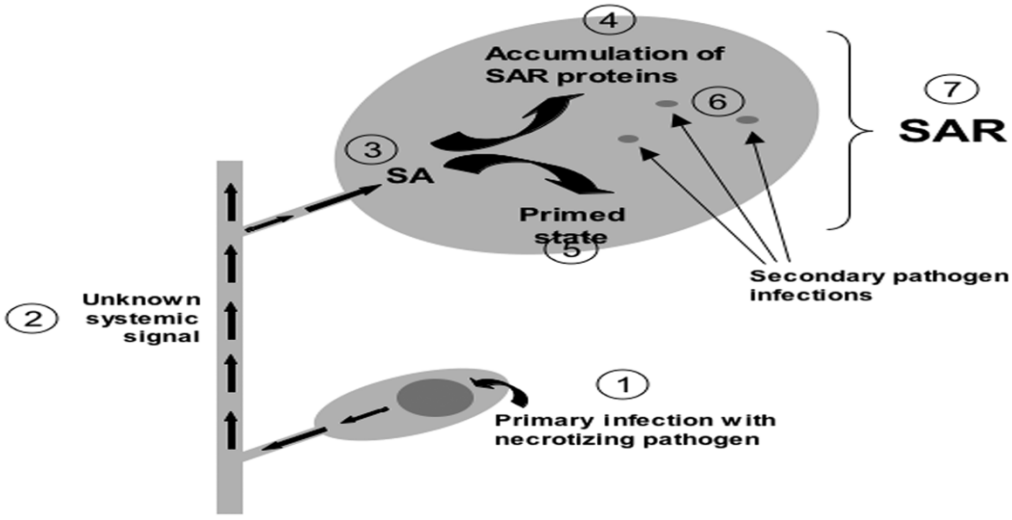


Fig. 3: A schematic diagram of the SAR response

Sequence of events associated with the establishment of SAR. Upon primary infection of a plant leaf with a necrotizing pathogen,

1. A yet unknown systemic signal(s) is distributed systemically throughout the plant.
2. The signal causes systemic accumulation of salicylic acid (SA).
3. SA causes direct activation of SAR genes.

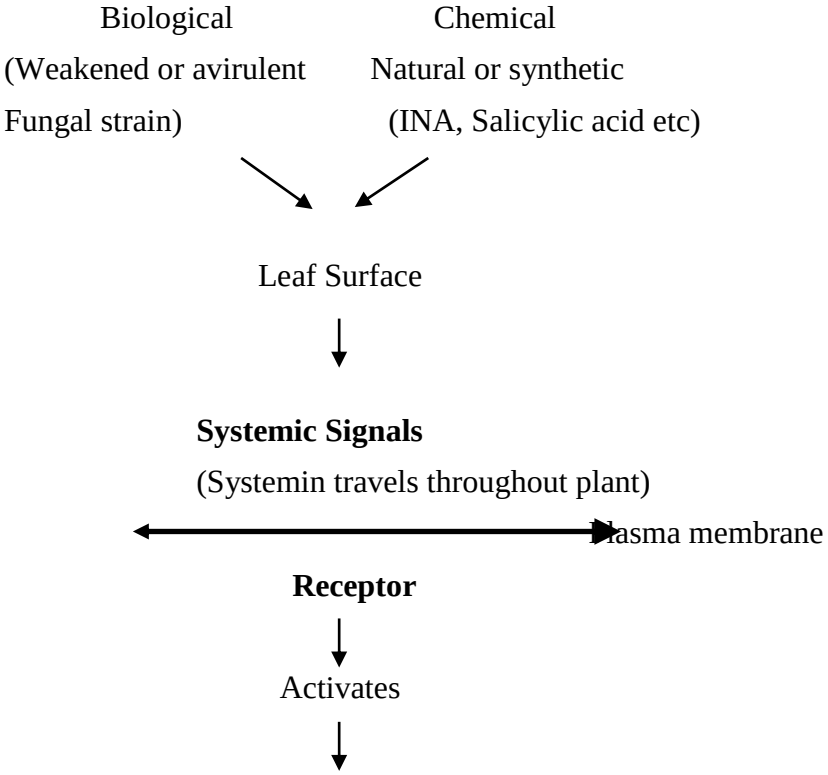
4. some of which encode enzymes with antimicrobial activity. SA also conveys the tissue to the primed state.
5. Which are characterized by an enhanced capacity to activate defense responses upon secondary pathogen attack. The faster and/or stronger activation of defense responses at the sites of secondary infection results in a decrease in disease symptoms, reflecting the SAR state.

NPR1: A key component for SAR

Numerous mutants with impaired SAR activation have been found over the past ten years. Among these mutations, the *Arabidopsis* mutant npr1 is most notable. In response to an infection with an avirulent pathogen, Npr1 builds up wild-type SA levels but is unable to activate *PR* genes, create the primed state, or produce biologically or chemically generated SAR. NPR1 is therefore probably a crucial regulator of SAR and priming. Two findings showing that constitutive overexpression of NPR1 in transgenic plants did not result in increased SA levels or constitutive *PR* gene expression provide additional support for this theory. Instead, these plants had significantly increased disease resistance as well as stronger PR gene expression following pathogen infection. Interestingly, *npr1* shows enhanced susceptibility to some virulent pathogens and seems to be involved also in R gene-mediated disease resistance.

Flow chart of SAR mechanism

SAR in plants



Lipase enzyme is switched on leading to the production of the fatty acid linolenic acid

↓

Linolenicacid Enzymes act to convert linolenic acid into a chemical derivative

↓

1, 2 OxoPhytoDienoic acid Enzymes act on chemical derivative to produce jasmonic acid

↓

Jasmonic acid and Methyl jasmonate

↓

Activates genes

Which result in biochemical and morphological changes

Biochemical	**Morphology**
Phytoalexin synthesis	Increased lignifications
Peroxidase synthesis	Enhanced papilla formation
Resin production etc.	Accumulation of cell wall protein

Long term resistance

Induced systemic resistance:

ISR signal transduction pathway

ISR needs ethylene and jamonic acid to signal. In addition to SA, the plant growth regulators ethylene and jasmonic acid (JA) have also been linked to the control of initial resistance responses in plants. In many situations, infection by a microbial pathogen is accompanied by increased synthesis of these hormones and concurrent activation of distant sets of genes involved in defence. An increase in resistance is frequently the outcome of these hormones being applied exogenously more and more.

We examined the expression of ISR in the *Arabidopsis* JA response mutant jar-1 and the ET response mutant etr-1. After *P. fluorescens* colonised the root, neither mutant was able to develop resistance to PST, indicating that ISR required JA and ET response. The efficacy of methyl jasmonate (MeJA) and 1- aminocyclopropane-1-carboxylate (ACC), the natural precursor

of ET, to induce resistance in wild type NahG jar-1 and etr-1 plants was studied in order to clarify the sequences of the signaling processes. In SA non-accumulating NahG plants, MeJA and ACC were successful in developing resistance against *P. syringe* pv. tomato suggesting that the SA independent ISR pathway is activated by both inducers.

The role of Jasmonic acid and Ethylene in ISR

Both jasmonic acid and ET activate particular groups of genes linked to defence in *Arabidopsis*, and when administered exogenously, they confer resistance to *P. syringe* pv. tomato. ISR connected to modifications in the expression of JA/ET sensitive genes. The *P. fluorescens* WSC 417r-mediated ISR expressed by the well-characterized JA/ET responsive gene (LOX-1, LOX-2, VSP, PDF 12, HEL, CHI-B, and PAL-1) in *Arabidopsis* plants. In induced plants, neither locally in the roots nor systematically in the leaves, none of the examined genes were upregulated. This shows that the level of JA/ET was not related to the resistance that was acquired.

Characterization of enhanced disease susceptibility mutants

In order to find potential new actors in the ISR signaling system, a collection of *Arabidopsis* eds mutants (enhanced disease susceptibility) to pathogen *P. syringe* bacterium was created. One of the 11 examined mutants, eds-1, eds-4, and eds-10, did not respond to P. fluorescens WSC 417r's induction of ISR. According to additional research on the ISR-impaired eds mutant, they are insensitive to the application of MeJA (eds1-1, eds4-1, and eds10-1) or ACC (eds4-1 and eds10-1) to induce resistance.

More specifically, Eds 4-1 and 8-1 demonstrated reduced expression of JA/ET responsive PDF. The 1.2 gene exhibits a normal responsiveness to both MeJA and ACC following treatment with MeJA and ACC, which reduced sensitivity to either ET (eds 4-1) MeJA (eds 8-1) or eds 10-1. Together, these findings showed that EDS-4, EDS-8, and EDS-10 are necessary for ISR and act either upstream of the JA/ET response (EDS-10) or in the ET response (EDS-10) in the ISR signalling pathway.

Mechanism of ISR

In this study, we attempted to clarify the stages involved in the SA-independent signaling cascade governing rhizobacteria-mediated ISR using well-characterized *Arabidopsis* mutants. The *Arabidopsis* mutants ***jar1, etr1***, and ***npr1*** prevented systemic resistance brought on by nonpathogenic rhizobacteria, showing that NPR1 and other elements of the jasmonate and ethylene response are essential for the ISR signaling cascade. As a result, the requirements for SA, on the one hand, and for jasmonate and ethylene, on the other, differ between the ISR signaling route mediated by rhizobacteria and the pathogen-induced SAR signaling pathway. We identified the series of signaling events involved in the route leading to resistance against P. s. tomato using MeJA and ACC as inducing agents. While ACC is completely active in jar1 plants,

MeJA-mediated protection against *P. s.* tomato necessitates an intact response to ethylene. Therefore, elements of the ethylene response function after jasmonate. Further evidence that NPR1 functions downstream of jasmonate and ethylene in the signaling pathway leading to resistance against *P. s.* tomato comes from the fact that MeJA- and ACC-induced protection is inhibited or significantly reduced in npr1 plants. Therefore, we hypothesise that during signal transduction that results in ISR mediated by *P. fluorescens* WCS417r, the ethylene and jasmonate responses are activated sequentially to cause a defence response that is controlled by NPR1. The discovery that NPR1-deficient plants had a partial suppression of ACC-mediated defence raises the idea of the existence of an additional, ethylene-inducible defensive pathway. The ethylene-inducible pathway leading to *Pdf1.2* gene expression, which has been demonstrated to be NPR1 independent, may be a possible pathway. Alternatively, the twofold increase in ethylene production following ACC treatment could be to blame for the poor level of protection in npr1 plants. Intriguingly, pathogen infection results in a considerably larger rise in ethylene synthesis in npr1 plants, indicating that the *npr1* mutation affects not just SA responsiveness but also ethylene metabolism.

The fact that *P. fluorescens* WCS417r, MeJA, and ACC all activate a similar SA-independent defensive mechanism in response to *P. s.* tomato infection suggests that ISR is linked to an increase in either ethylene or jasmonate synthesis. However, jasmonate and ethylene-responsive gene expression do not overlap with *P. fluorescens* WCS417r-mediated ISR, indicating that the production of these compounds is not significantly promoted. Without inducing Atvsp, Hel, or Pdf1.2 gene expression, plants exposed to lower doses of MeJA or ACC (25 mM and 0.25 mM against 100 mM and 1 mM, respectively) exhibited increased resistance to *P. s.* tomato. A mild or limited stimulation of the jasmonate and ethylene response, below the threshold level required for *Atvsp, Hel,* and *Pdf1.2* gene activation, may therefore be a component of *P. fluorescens* WCS417r driven ISR. As a result, ethylene and jasmonate-dependent plant responses can be induced without these phytohormones' levels also rising. Elucidating the role of increased sensitivity to ethylene or jasmonate in rhizobacteria-mediated ISR is necessary. Independent of SA, pathogen-induced systemic activation of the *Arabidopsis* plant defensin gene Pdf1.2 necessitates elements from both the ethylene- and jasmonate-response pathways. As a result, this defensive response and the WCS417r-mediated ISR of P. fluorescens appear to share some unique signaling processes.

ISR is dependent on NPR-1:

It has been demonstrated that NPR-1 is an independent signaling component of the SA-dependent SAR response. The ability of mutant npr-1 to induce ISR in Arabidopsis was examined. Unexpectedly, mutant npr-1 plants were unable to express *P. fluorescens* WSC 417r

driven ISR, suggesting that pathogen-induced SAR is similar. A defence reaction that depends on NPR-1 is RISR.

The order of ISR signaling events was clarified, and it was discovered that NPR-1 functions in the ISR signaling pathway after JA and ET. Evidently, NPR-1 requires not only the SA-dependent activation of PR genes but also a defence response that is mediated by rhizobacteria and reliant on JA and ET. These show that the NPR-1 gene can variably influence the expression of defensive genes. Finding the signaling elements from the ISR and SAR pathway that confer the specificity on NPR-1 dependent defence gene activation is one of the biggest hurdles.

***P. fluorescens* WCS417r-mediated ISR in Arabidopsis**

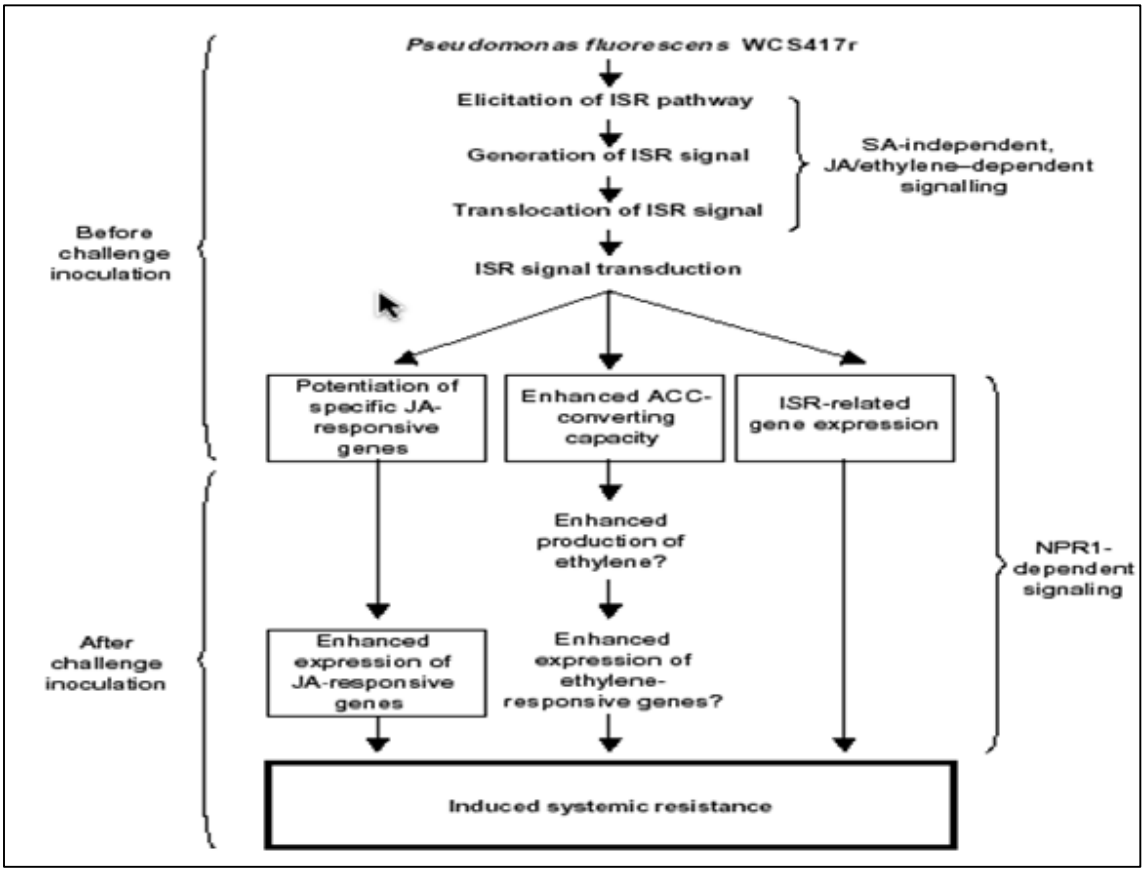


Fig. 4: Working model is explaining the possible involvement of JA and ethylene in *P. fluorescens* WCS417r-mediated ISR in Arabidopsis

In contrast to the pathogen-induced SAR pathway, which depends on SA and is linked to the activation of PR genes, WCS417r colonisation of *Arabidopsis* roots initiates a signaling pathway that needs responsiveness to both ethylene and JA. The JA and ethylene response components are successively activated in the ISR pathway, resulting in a systemic resistance that, like SAR, is controlled by NPR1. It is possible that this signal molecule is involved in the creation or translocation of the systemically delivered signal because ethylene responsiveness is required at the site of ISR induction. This does not exclude the likelihood that ethylene and/or JA

are also involved in the ISR pathway's later phases, though. ISR is not followed by a rise in ethylene or JA production either locally or systemically. Therefore, in response to activation of ISR, *Arabidopsis* plants do not exhibit an increase in JA- or ethylene-responsive gene expression. But in ISR-expressing plants, a particular group of JA-responsive genes is potentiated, resulting in a higher degree of expression after challenge inoculation. Additionally, ISR-expressing plants are better able to convert ACC to ethylene, which increases their capacity to produce ethylene in response to a challenge. Both results imply that ISR-expressing plants are prepared to express ethylene- and/or JA-dependent defence reactions more quickly or to a higher level in response to an aggressive pathogen attack.

Factor affecting induced resistance:

- Signaling pathways can interact in a positive or negative way. For instance, (a) synergistic activities against *P. syringe* pv. tomato in *Arabidopsis* SAR and ISR. (b) ASM (activator of SAR) treatment increased the sensitivity of tomato leaves to herbivorous caterpillars. (Pieters *et al*., 1999.)
- Different cultivars react differently to particular activators' induction resistance. Examples include (a) Host genotypic diversity in ASM's ability to cause resistance in cucumbers to the hemi-biogenic pathogen *Colletotrichum orbiculare*. (b) When the same cultivar is exposed to the disease *Didymella bryoniae* and induced with ASM, some of it exhibits resistance, while some respond slowly and others become more vulnerable. (Richa and Hammerschmidt).
- The defendant plant benefits from induced resistance since it reduces illness, but occasionally the plant must pay a price for the resistance. After the use of elicitors, there may be a noticeable diversion of energy and carbon precursors away from other crucial processes, which may lead to the accumulation of phytoalexins.
- Reduction of disease by mechanism of SAR/ISR may not be necessary accompanied by better quality and increased in yield. E.g. Application of JA affects fruit setting. (Delay in fruit set, formation of larger fruit but few in number, increased in ripening time, fewer seeds per fruit per tree)

Conclusion:

Regarding the application of IR in agriculture, ecological research has the ability to go beyond simply interpreting the occurrence to provide a deeper understanding of the ecology and evolution of induced pathogen resistance. Possible issues arising from ISR allocation costs or ecological costs (negative effects on, for example, mutualistic microbes or insects) must be taken into account and carefully investigated when IR is to be used in crop protection by either chemical elicitation or transforming plants to exhibit stronger or faster IR, or to express IR compounds constitutively.

To accurately assess the danger associated with this new, "integrative," plant protection technology, research on ecologically realistic study systems and ecologically designed studies in agricultural systems are thus required. Induced resistance in plants, though still poorly understood, offers fresh perspectives on plant defence and holds promise as a tool for sustainable farming and environmentally friendly disease control. It continues to be difficult for both basic and practical research.

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Chapter 2 MOLECULAR BASIS OF PLANT PATHOGEN INTERACTION

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Abstract:

Agriculture is constantly at danger from plant disease all around the world. Creating a sustainable agriculture requires an understanding of how pathogens and plants interact. Plant immune response has resulted in a strong defence system that is able to withstand possible attack by microbial diseases due to the long history of co-evolution between hosts and pathogens. Plants must rely on their innate immunity, which is present in every cell, and on systemic signals coming from infection sites because they lack mobile defence cells and somatic adaptive mechanisms. Through receptors, a surveillance system that can identify both conserved chemical patterns and particular effector proteins and activate the relevant defences, the pathogen is perceived. PAMP-triggered immunity (PTI), which is the name of the initial immune response, must develop to recognise the typical characteristics of microbial infections. In order to repress PTI and promote pathogen growth and disease, pathogens developed the ability to transfer effector proteins to the plant system at the same time. Plants developed surveillance proteins (R proteins) as a result, which they use to either directly or indirectly detect the presence of pathogen effector proteins or activate the defence cascade. The way we think about pathogenesis and the management of diverse plant diseases is evolving as a result of the new insights in the finding of the molecular basis of host pathogen interaction. We will be able to develop new techniques for managing plant diseases if we use an integrated strategy based on the knowledge of the molecular underpinnings of both the "defensive systems" utilised by plants and the "attack systems" used by pathogens.

Keywords: Molecular basis, pathogens, plant diseases, host pathogen.

Introduction:

Because they are sessile organisms, a variety of parasites, such as viruses, bacteria, fungi, nematodes, insects, and even other plants, frequently use plants as a source of food and refuge. The main factors that determine whether a plant is susceptible to a pathogen attack or resistant to it are interactions between disease resistance (R) genes in plants and their corresponding pathogen avirulence (Avr) genes. There is growing evidence that these gene-for-gene interactions play a role in plants' sensing of pathogenic incursions and acquisition of acquired resistance through a variety of still poorly understood molecular and hormonal transduction pathways. It has been discovered that before the overexpression of R genes, plants actively create a number of phytohormones, including ethylene, jasmonate, salicylic acid, and reactive oxygen intermediates. The generation of mutants with changed R genes and the use of transgenic plants

in recent experiments have revealed new details about the mechanisms underlying pathogen recognition, signal transduction, and consequent disease resistance in plants. The complexity of the biological organisation and the specificity of the interactions between plants and diseases remain an enigma. A significant issue in modern plant pathology is to explain this phenomena. However, when the entire sequencing of the molecular research model Arabidopsis plant genome becomes a reality, there are expected to be enormous prospects for crop enhancement. Numerous R genes have been discovered, and they protect different plant species from a wide variety of diseases.

Interactions between host and pathogen

Plants typically respond to diseases with resistance, while susceptibility is a rare occurrence. In order to defend themselves against bacteria that are continuously in contact with their potential host, plants have evolved powerful defence systems. Pathogens must encounter and overcome a variety of barriers on their journey into the plant tissue in order to spread illness. The plant cell surface serves as the first barrier. Natural holes like stomata, open wounds, and direct penetration employing enzymes and/or mechanical forces are also possible routes for penetration. The second barrier, the plant cell wall, is encountered by pathogens once they have pierced the plant cuticle. After breaking through the cell wall, only the plasma membrane keeps the pathogen from the plant's cytoplasm. Extracellular surface receptors, specialised proteins found in plasma membranes, are important in the detection of pathogen-associated molecular patterns (PAMPs) in order to initiate immune responses. One of the main fungal PAMPs is chitin, which is a component of cell walls. Both inherent and induced defensive mechanisms are present in plants. The former comprises inherent characteristics like cell wall strength and thickness as well as the existence of naturally occurring antibacterial substances like polyphenol. On the other side, induced resistance involved physically impassable barriers and novel harmful substances. A multi-part defence response whose elements are triggered in a highly controlled temporal and geographic way is started by regulatory genes upon pathogen detection. The production of phytoalexins, the buildup of (hydrolytic) pathogenesis-related (PR) proteins, the activation of signalling pathways, and the reinforcement of the cell wall, the production of reactive oxygen species, and finally programmed cell death and systemic acquired resistance are typical elements of such responses (SAR). When defence responses are triggered or activated in compatible contacts, the pathogen is able to exercise its detrimental effects on the plant's growth and development.

How pathogens recognize host plant?

Typically, a plant's reaction to a disease depends on its capacity to identify a hallmark molecule the infection produces. The majority of these molecules, known as exogenous elicitors, come from the pathogen and include peptides, glycoproteins, lipids, and oligosaccharides.

Additionally, when a pathogen attacks a plant, endogenous elicitors are released from the plant's surface. The pathogen's avirulence gene encodes elicitors that are particular to racial groups and cultivars. Leucine rich repeat (LRR) domains enable the majority, but not all, proteins generated by the R gene to recognise Avr proteins and to start signalling through interactions with kinases or through kinase domains. LRR receptor kinase and a mitogen-activated protein (MAP) kinase cascade are components of a signal transduction pathway for bacterial flagellin that activates specific transcription factors. They have been divided into many groups based on their structural properties, including histidine kinase receptors, receptors with variable numbers of transmembrane domains, and receptors that are similar to receptor-like protein kinases (RLKs). The RLKs, of which the Arabidopsis genome contains at least 340 genes encoding putative RLKs, are of particular importance in pathogen perception. The extracellular domain of RLKs, which is likely involved in signal detection, the transmembrane domain, and the cytoplasmic kinase domain, which may start a signal transduction cascade inside the cell, are the three primary features of RLKs. All of the discovered plant RLKs are serine-threonine kinases, and they have been classified into various groups based on the structural characteristics of the extracellular domain.

Molecular basis of Host Pathogen recognition

One makes use of flagellin-responsive transmembrane pattern recognition receptors (PRRs), which react to slowly evolving MAMPS or PAMPs (microbial- or pathogen-associated molecular patterns). Using the polymorphic NB-LRR protein products that are produced by the majority of R genes, the second mostly functions inside the cell. Their distinctive nucleotide binding (NB) and leucine rich repeat (LRR) domains gave rise to their names.

Modes of plant pathogen interactions

Direct Interaction

In order to genetically explain the great specificity of plant-pathogen interactions, the "gene-for-gene" model was put out. This model predicts that there will be a receptor/ligand-like interaction between the products of plant R genes and the matching pathogen-derived Avr genes. A few instances of direct interaction between Avr proteins and R proteins, such as Pto-AvrPto, Pita-AvrPita, RRS-1R, and popP2, have been shown. RIN4 adversely controls a plant's basal defence, which PAMPs activate through the appropriate receptors (e.g. FLS2 for flagellin). Through the TTSS, the effector AvrRpm1 or AvrRpt2 is introduced into an Arabidopsis cell. The phosphorylation of RIN4 that results from AvrRpm1 binding to RIN4 may increase RIN4's capacity to decrease basal defence through an unidentified mechanism. AvrRpt2 cuts RIN4 in half. Unless the cleavage products are stronger suppressors, it is unknown how cleavage of RIN4 might improve the suppressor function. AvrRpt2 may reduce defence through additional ways because it targets multiple host proteins.

Indirect interactions (Guard Hypothesis)

The link between the LRR domain of the rice Pi-ta CC-NBS-LRR protein and the Avr-Pita protein of the rice blast fungus *Magnaporthe grisea* was previously the only example of a direct interaction between an NBS-LRR protein and a pathogen Avr determinant. The 'guard' hypothesis, which contends that the interaction between a R protein and its cognate Avr determinant is mediated by a host protein that is the target for the effector function of the Avr determinant on the one hand and under R-protein surveillance for such interference on the other, was developed in response to the lack of evidence for direct interaction in many other systems examined. The protein RIN4 (RPM1 interacting) from *Arabidopsis* is one of the examples providing compelling evidence for the "guard" theory. RPM1 and RIN4 interactions are mediated by RIN4 (resistance to *P. syringae pv. maculicola*). Between the RPS2 (resistance to *P. syringae* pv. tomato) CC-NBS-LRR protein and the AvrRpt2 type III effector protein, also from *P. syringae*, on the one hand, and the AvrB and AvrRpm1 type III effector proteins from the leaf speck bacterium *P. syringae* on the other. RIN4 forms a complex with RPM1 and RPS2. RIN4 is phosphorylated by AvrB and AvrRpm1, which activates RPM1, while RIN4 is degraded by AvrRpt2, which inhibits RPM1's ability to function but activates RPS2.

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Chapter 3 MECHANISM OF INDUCED SYSTEMIC RESISTANCE (ISR) IN PLANTS

Nitisha Gahlot, Shaik Munnysha, Ramavath Abhi, Manisha

Abstract:

In response to biotic stressors (pathogens and parasites) of different sizes, plants have a variety of active defensive mechanisms that can be actively expressed (ranging from microscopic viruses to phytophagous insect). The timing of this defensive reaction is crucial and determines whether an organism can overcome the biotic challenge posed by necrotizing diseases and parasites or not. Disease can be lowered if defensive mechanisms are activated by a stimulation before a plant pathogen infects the plant. When a plant is sufficiently stimulated, it might acquire an improved defensive capacity known as induced resistance. Two types of induced resistance—induced systemic resistance (ISR) and systemic acquired resistance (SAR)—involve preconditioning plant defences through prior infection or treatment, which confers resistance against a pathogen's later challenge. Selected strains of plant growth-promoting rhizobacteria (PGPR) fight off soil-borne pathogens, causing a systemic resistance in the plant against both root and foliar pathogens, and suppressing illness. In that both types of induced resistance make uninfected plant portions more resistant to a wide range of plant diseases, rhizobacteria-mediated ISR is similar to pathogen-induced SAR. By generating salicylic acid (SA) at the root surface, a number of rhizobacteria activate the salicylic acid (SA)-dependent SAR pathway, whereas other rhizobacteria activate a distinct signaling route without the need for SA. In Arabidopsis thaliana, which is dependent on jasmonic acid (JA) and ethylene signaling, the existence of an ISR route that is not dependent on SA has been explored. Certain Pseudomonas strains cause systemic resistance in several plant species, including Arabidopsis, cucumber, radish, and carnations, as evidenced by an enhanced defensive capacity upon challenge inoculation. In addition to extending protection to a wider range of pathogens than ISR/SAR alone, the combination of ISR and SAR can improve protection against infections that are resistant through both pathways. Along with Pseudomonas strains, Bacillus spp. also carry out ISR, and published results demonstrate that a number of particular strains of the species *B. amyloliquifaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides*, and *B. sphaericus* significantly reduce the incidence or severity of a variety of diseases on a variety of hosts.

Keywords: Induced systemic resistance, Jasmonate, ethylene signalling, Pathogenesis-related proteins.

Introduction:

Select strains of non-pathogenic, root-colonizing, plant growth-promoting rhizobacteria (PGPR) were demonstrated to cause systemic resistance in carnation and cucumber in 1997 (Van hem et al), presenting a novel type of induced resistance. Van Loon designated the PGPR-induced resistance as induced systemic resistance (ISR) to separate it from the pathogen-induced SAR that is responsible for this improved defence capacity (1997). Pseudomonas rhizobacteria, non-pathogenic bacteria that colonise plants' roots, cause systemic resistance in plants, which, unlike SAR, doesn't require tissue necrosis, salicylic acid signal molecules, or PRP production. The signalling molecules for ISR, ethylene and jamonic acid, operate sequentially to activate the induced systemic resistance response. They are known as PGPR bacteria because they increase plant growth and yield by suppressing deleterious rhizobacteria (DRB). Rhizobacteria added during seed bacterization using PGPR colonise newly developing roots and shoots. Once ISR has been induced, the quantity of injected bacteria may decrease without losing their ability to protect against DRB. In addition to seed bacterization, other methods for generating ISR include directly applying PGPR to roots during transplantation or putting seedlings in bacterized soil.

Rhizobacteria-induced ISR has been demonstrated against fungi, bacteria and viruses, in Arabidopsis, bean, carnation, cucumber, radish, tobacco and tomato, but not in any monocot plant.

The exposure of the plant to pathogenic, avirulent, and nonpathogenic microorganisms can cause SAR. A specific amount of time is needed for the creation of SAR, during which pathogenesis-related proteins (chitinase and glucanase), as well as salicylic acid, accumulate depending on the plant and elicitors. Plant growth-promoting rhizobacteria (PGPR), of which strains from the genus Pseudomonas that do not obviously harm to the plant's root system are the best characterised, enhance ISR. ISR, in contrast to SAR, relies on pathways controlled by jasmonate and ethylene rather than accumulating pathogenesis-related proteins or salicylic acid.

Induced-resistance systems in plants

Induced resistance in plants is a tremendously complex system that has only been partially understood in a number of model plant species, including Arabidopsis. In Arabidopsis, there are three generally accepted pathways of induced resistance, two of which are associated with the direct production of pathogenesis-related (PR) proteins. In one pathway, the production of PR proteins is typically triggered by an attack by pathogenic microorganisms, whereas in the other, PR proteins are typically triggered by wounding or necrosis-inducing plant pathogens. Both pathways, however, have alternate mechanisms for induction. The wounding pathway typically uses jasmonic acid (JA) as the signalling molecule, whereas the pathogen-induced pathway typically uses salicylic acid (SA), which is produced by the plant. When administered exogenously, these substances and their counterparts produce comparable effects, suggesting that

there is likely significant cross-talk between the pathways. Induced systemic resistance (ISR), the name given to the JA-induced pathway, is also used to describe several distinct processes that are started by rhizobacteria. Production of a cascade of PR proteins, including antifungals like chitinases, glucanases, and thaumatins, and oxidative enzymes like peroxidases, polyphenol oxidases, and lipoxygenases, respectively, characterises the salicylate and jasmonate-induced pathways. Phytoalexins, low-molecular weight molecules with antibacterial capabilities, can also build up. The third type of induced resistance is known as rhizobacteria-induced systemic resistance (RISR), which was responsible for the evolution of antibiotic resistance. The PR proteins and phytoalexins are not produced by rhizobacteria colonising roots when plant-pathogenic microbes are not present, therefore it is functionally very different. When a plant is attacked by a pathogen, its defences are stronger and the disease is less severe. Thus, in the absence of the protein cascade that is typical of the SA-induced system, RISR causes a potentiation of plant defence responses.

Arabidopsis as a model to study Rhizobacteria-Mediated ISR

An Arabidopsis-based model system was created in order to study rhizobacteria-mediated ISR because this plant species has been thoroughly examined for molecular genetic research on plant-microbe interaction, using the non-pathogenic rhizobacterial strain Pseudomonas fluorescens WCS 417r as an inducing agent. The bacterial leaf pathogens *Ps. syringae* pv. tomato and *Xanthomonas campestris pv. armoraciae,* the fungal root pathogen *Fusarium oxysporum,* the fungal leaf pathogen *Alternaria brassicicola,* and the oomycete leaf pathogen *Peronospora parasitica* are just a few of the pathogens that are protected from by the colonisation of *Arabidopsis* roots.

Role of ISR

Plant roots are thought to be connected to microbial populations in suppressive soils that generally benefit plant health. In fact, a number of biocontrol PGPR induce ISR in the host plant, allowing plants to resist pathogen attack on the leaves or roots without providing complete protection. ISR is induced by several efficient biocontrol PGPR, regardless of antibiotic synthesis. Through transcriptome (expressed level of proteins) investigation of plants with roots that were colonised by one of these strains, the effects of three different strains of *Pseudomonas spp*. mediating ISR in *Arabidopsis thaliana* have been examined (*P. fl uorescens* WCS 417r, P. thivervalensis and P. fluorescens CHA0). When compared to the uninoculated control and the systemic reactions that are generally observed after attack by necrotizing pathogens, the transcript levels in the leaves were not noticeably altered in any of the cases, i.e., they varied by less than a factor of three.

The mechanism of rhizobacteria-induced systemic resistance (RISR)

Since numerous pathogens can be averted in natural settings, the largely non-specific nature of IR results in a gain in basal resistance to various pathogens concurrently. Understanding the bacterial plant mechanisms underlying the rhizobacteria-mediated ISR phenomenon is crucial, as is deciphering the prerequisites for ISR induction, signalling, and expression.

Induction of ISR

ISR-triggering rhizobacteria must produce different eliciting components from pathogen elicitors because beneficial rhizobacteria do not obviously harm their host or induce localised necrosis. The bacterial components that cause ISR are rather little understood. Similar components are present in microorganisms that appear to be recognised by eukaryotic cells; the mechanism of elicitation shares several similarities with the generation of specific non-specific defence reactions in plant cells in response to general pathogen-associated molecular patterns (PAMPs). LPS and flagella, which are present on cell surfaces, can set off a defense-related reaction in suspension-cultured plant cells and leaves. Following challenge inoculation of treated plants with the causative agent of bacterial speck disease, both of these components of the rhizobacterial strain WCS 358 have the capacity to induce ISR when administered as pure components to the root system of Arabidopsis plants. Similar to plants grown in soil containing the wild type strain WCS 358, the pathogenic bacterium *P. syringae pv*. tomato (Pst), which causes chlorotic and necrotic signs on the plants, was significantly reduced.

Signalling in pathogen-induced SAR

Mutant and transgenic plants have been used to study the identification of crucial steps in the signal transduction pathway for SAR. When SA was shown to be an endogenous chemical in plants that grew in amount during elicitation, it was confirmed that a phenolic compound structurally similar to SA was necessary for the creation of SAR. Recently, it has been postulated that when SA is induced, local levels rise, which is linked to the formation of a mobile signal that travels throughout the plant and starts additional local SA production in distant leaves. For the systemically induced condition to be conferred, this level of SA is both necessary and sufficient.

It has not been determined how SA causes resistance or what causes the plant to produce more SA, nor is it known what triggers this increased SA production. Animal innate immunity is influenced by the protein NPR1, a member of the ankyrin-repeat family that structurally resembles the inhibitor of IF-kB required for SA activity in plants. Under the influence of SA, a redox shift induces NPR1 oligomers in the cytoplasm to dissociate into monomers. These monomers are carried into the nucleus, where they interact with certain TGA transcription factors to enable the production of genes for proteins involved in disease (PRs). These findings gave rise to the theory that the existence of PRs is necessary for the status of SAR.

Signalling in rhizobacteria-induced systemic resistance (RISR)

ISR signaling seems more intricate than SAR signaling. Several ISR-eliciting rhizobacterial strains have been identified, some of which can also produce SA and some of which cannot. This can be explained using two criteria: (1) The ISR should be linked to the induction of PRs, and (2) in Nah G plants, both the ISR and the induction of PRs should be eliminated (SA deficient). Upon challenge inoculation with 7NSK2, ISR against Botrytis cinerea and Tobacco Mosaic Virus (TMV) is eliminated in tobacco and tomato plants, and in Arabidopsis against *P. syringae pv. maculicola* following elicitation by *B. pumilus* SE342. In contrast to other rhizobacterial strains that can produce SA in vitro, such as WCS 374 on *Arabidopsis*, which otherwise elicits ISR in an SA-independent manner, Serratia marcescens on tobacco or *P. fluorescens* CHA0 on Arabidopsis, strain WCS 358, which does not produce SA, elicits ISR in *Arabidopsis*. This data suggests that rhizobacterial production of SA.

Bacterial determinants of induced systemic resistance in different plant species

Bacterial strain	Plant species	Determinant
B. amyloliquefaciens IN 937a	Arabidopsis	2,3-butanediol
B. subtilis GB03	Arabidopsis	2,3-butanediol
P. aeruginosa 7 NSK2	Bean	SA
P. fluorescens CHA0	Arabidopsis	SA
P. fluorescens Q2-87	Arabidopsis	2,4 DAPG
P. fluorescens WCS 374	Radish	LPS
P. fluorescens WCS 417	Arabidopsis, Carnation and Radish	LPS
P. putida WCS 358	Arabidopsis Bean Tomato	LPS, Siderophore, Flagella LPS, Siderophore LPS, Siderophore
P. fluorescens GRP3	Rice	Siderophore
Rhizobium etli G12	Potato	LPS
Serratia marcescens 90-166	Tobacco	Fe-regulated compounds

ISR expression Upon challenge inoculation with a pathogen, ISR expression is comparable to SAR in that disease severity and the number of infected plants both decrease. This lowering affects the plant's capacity to fend off the disease by reducing the pathogen's development and colonisation of the induced tissues. Due to differences in defensive signals, the range of disorders that ISR and SAR are useful in treating only partially overlap. Pathogens can be thwarted in Arabidopsis by defences that are either SA-dependent, JA- and/or ethylene-dependent, or both. In both locally and systemically generated resistance responses, SA plays a

key signaling role; however, studies on ISR signalling mediated by rhizobacteria have shown that JA and ethylene play the major roles28. In this way, ISR expression resembles SAR in terms of phenotype and depends on other defense-related processes in addition to a different kind of biological induction. ISR against Pst is expressed normally in mutants of *Arabidopsis* that are impaired in the synthesis of the phytoalexin camalexin (pad1-pad4), indicating that ISR does not function through stimulation of phytoalexin production. Plant defence molecules, such as phytoalexins, can also contribute to plant resistance.

While ISR is more active against nectrotrophic pathogens in *Arabidopsis*, SAR is more effective against biotrophic pathogens, including as viruses and downy and powdery mildews that are vulnerable to SA-dependent responses. SAR was previously found to be ineffective against the two most common necrotrophic fungi, Botrytis cinerea and *Alternaria brassicicola*. The efficiency of SAR and ISR against various pathogen types in tobacco is essentially comparable to their varied actions in *Arabidopsis*. However, SA-dependent responses against Botrytis were reported to be implicated in tomato's resistance to the powdery mildew fungus Oidium neolycopersici. This leads to the conclusion that different pathogens can be successfully combated by various plant species via SA, JA, or ethylene-dependent defensive mechanisms.

When challenged with Pst, it was shown that SAR-induced plants displayed increased expression of the SA-dependent PR-1 mRNA, and ISR-induced plants accumulated more JA-inducible gene vsp mRNA than non-induced plants. This "priming" effect demonstrated that defense-related gene expression is more rapidly and intensely activated in induced plants than in non-induced plants. By using subtractive hybridization, it was discovered that the ISR generated by *P. chlororaphis* O6 upon target leaf spot inoculation against *Corynespora cassiicola* was linked to a faster and stronger accumulation of transcripts for six different genes.

Systemically induced resistance (SIR) and plant growth

Both SA-dependent SAR and JA- and ethylene-dependent ISR and SIR must be expressed by an augmented inoculation-induced activation of the immune system. While SAR is linked to the buildup of PRs and has a detrimental effect on plant growth, the majority of rhizobacteria that generate ISR have been chosen for their abilities to promote plant growth. In addition to triggering ISR, PGPR can guard against soil-borne diseases that are particularly aggressive against newly emerging seedlings. Plant vigour is undoubtedly boosted by stimulating plant development. Rhizobacteria that induce ISR can be given to seeds, whereby they quickly colonise growing plant roots and improve seedling protection.

Conclusion:

To lessen diseases brought on by microorganisms that are susceptible to JA- and ethylene-dependent defences, ISR-inducing PGPR is a valuable strategy. It will be worthwhile to

investigate the integration of ISR-triggering PGPR into disease management programmes in conjunction with other techniques.

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Chapter 4 RESISTANCE GENES (R-GENES) IN PLANT DEFENSE MECHANISM

Manisha, Shaik Munnysha, Ramavath Abhi and Nitisha Gahlot

Introduction:

R-genes are genes that provide pathogen resistance to plants by generating R-proteins, and they are found in plant genomes. When a pathogen's matching avirulence genes are expressed, disease resistance genes (R genes) in the host plant are also expressed. Following the gene for gene principle

Resistance in hosts and avirulence in pathogens

Alleles of the relevant plant disease resistance (R) locus interact specifically with the pathogen avirulence (avr) gene loci to control plant-pathogen interactions, particularly those involving biotrophic parasites. Disease resistance results from the presence of the matching R and avr genes in both the host and the pathogen. Disease develops if either is inactive or absent1. According to the simplest model that explains this genetic relationship, R products must recognise avr-dependent signals and start a series of signal-transduction events that result in the activation of defence systems and the cessation of pathogen growth. A polymorphism element of a specific recognition event is specified by the R gene. One or more basic defence pathways are overlaid with specific R-mediated innate immunity. After successful infection and the development of disease, fundamental defences prevent pathogen spread. The discovery of mutants that are more vulnerable to a virulent virus than their parents is used to imply the existence of basal defence (detailed below). It is possible that one purpose of R-mediated signalling is to more quickly and efficiently activate defence mechanisms that are shared by both pathways. This idea is supported by genetic overlap between specialised and basal resistance responses.

The functional R genes that have been discovered so far encode resistance to pathogens that live outside or inside the plant cell and include bacteria, viruses, fungi, bacteria, oomycetes, even nematodes and insects. R genes only encode five types of proteins, despite the enormous variety of pathogen taxa and their hypothesised pathogenicity effector molecules. Nucleotide binding site plus leucine-rich repeat (NB-LRR) class of proteins are encoded by the majority of R genes.

Structural domains of resistant gene products

Nucleotide Binding Sites

In addition to encoding LRRs, several resistance genes also produce amino acid sequences that are very similar to recognised nucleotide binding sites. The ATP synthase p

component, ras proteins, ribosomal elongation factors, and adenylate kinases are only a few examples of the many proteins with ATP or GTP binding activity that contain these NBS domains. Some R gene products have the highly conserved NBS domain, which shows that nucleotide triphosphate binding is crucial to the operation of these proteins.

Leucine-Rich Repeats

LRRs are numerous, serial repeats of a motif that is 24 amino acids long. Regularly spaced prolines and asparagines can also be found in LRRs, along with leucines or other hydrophobic residues. Porcine RNase inhibitor, one LRR-containing protein, has had its crystal structure established. The LRRs in this protein produce a tertiary structure that resembles a fist or a bent spring, with each curled finger denoting a single LRR. These repetitions are unusually lengthy in porcine RNase inhibitor (28 to 29 amino acids each). Individual repeats for many LRR-encoding sequences that have been reported frequently contain residues that do not match the consensus LRR residues, and in some cases this degeneracy is enough to imply a deviation from a highly regular structure. In addition, the postulated LRR region of some R gene products is bisected by a short stretch of amino acids that appears unlikely to form an LRR structure.

Serine-Threonine Kinases

The cloning and characterization of Pto showed how crucial signal transduction by kinases is for gene-for-gene plant disease resistance. One of the most prevalent methods used by living things to regulate protein function is phosphorylation state modulation, and protein kinases have received a lot of attention. These conserved domains are present in the Pto gene's derived amino acid sequence and Pto has been demonstrated to have in vitro protein kinase catalytic activity (Loh and Martin, 1995; Rommens *et al*., 1995a). Pto has a possible myristoylation site at its N terminus that might serve as a membrane anchor for this predominantly hydrophilic protein (Martin *et al*., 1993). Serine-threonine kinase-related discoveries included the identification of Fen, Ptil, and Xa21.

Leucine Zippers

There are several subgroupings of R genes within the NBS-LRR subclass. Three genes, RPS2, RPM1, and Prf, all likely encode leucine zipper (LZ) sequences between the N terminus and the NBS and LRR domains. These genes are resistant to *P. syringae* pathovars. These heptad repeat sequences promote the development of coiled-coil structures in other proteins, facilitating protein-protein interactions (Alber, 1992). Although comparable coiled coil domains promote connections between proteins with a variety of other functions, LZs are best known for their role in homo- and heterodimerization of eukaryotic transcription factors.

Toll-lnterleukin-1 receptor

Similarity In that they encode a sizable N-terminal region identical to the cytoplasmic signaling domain of mammalian interleukin-l receptors and the Drosophila To11 protein, N and

L6 comprise a second class of NBS-LRR R genes (Whitham *et al*., 1994; Lawrence *et al*., 1995). Recently, the Arabidopsis RPf5 R gene was also cloned and added to this group of R genes.

Classification of R genes

Five classes of disease resistant genes defined according to structural characteristics

Class 1:

The Hm1 gene from maize was the first R gene to be cloned by Johal and Briggs in 1992. The Flor's gene for gene hypothesis is not supported by the Hm1 gene. It contains the coding for an enzyme that detoxifies the HC toxin produced by *Cochliobolus carborum* (*Helminthosporium maydis*). Race 1 is an example of the first class of R genes, which differs from real R genes in that they recognise a particular pathogen race to initiate a defence response.

Class 2:

Kinases class is the second category of resistance gene. Intracellular serine/threonine-specific protein kinases are encoded by this class. It contains the tomato Pto gene, which was cloned by Martin *et al*. in 1993 and is efficient against *Pseudomonas syringae* pv. tomato. The first cloned race-specific R gene that complied with the gene for gene recognition was this one. The downstream signal transduction that triggers the hypersensitive response is made possible by Pto kinase.

Class 3:

It encodes cytoplasmic receptor like proteins that contain an NBS cascade and LRR. This class is further divided into 2 subclasses.

Class 3a (LZ+NBS+LRR):

It includes *RPS2* and *RPM1* from *Arabidopsis* against *P. syringae* pv. *Tomato*. It is suggested that the LZ (Leucine Zipper) participates in the homo- and heterodimerization of eukaryotic transcription factors. Proteins with NBS (Nucleotide binding sites) are required for a variety of key eukaryotic cellular processes.

Class 3b TIR + NBS +LRR:

It includes N gene from tobacco, L6 from flax and RPP5 from Arabidopsis N is operative against tobacco mosaic virus, L6 against *Melampsora lini, RPP5 against Peronospora parasitica*. The presence of TIR domain in amino acid terminal of R genes indicates that this domain may trigger signal transduction pathways

Class 4 LRR+TM:

These genes from the tomato, Cf2, Cf4, Cf5, and Cf9, condition resistance to *Cladosporium fulvum*. These have extracellular LRR domains and TM (Transmembrane receptors) motifs.

Class 5 LRR Kinase family:

Xa21 gene from rice confers resistance against *Xanthomonas oryzae* pv. *oryzae* race 21.

Classification of plant disease resistant genes

Class	Gene	Plant	Pathogen	Predicted features of R gene
1	Hm1	Maize	*Helminthosporium maydis*	Detoxifying enzyme HC- reductase
2	Pto	Tomato	*Pseudomonas syringae*	Intracellular ser/thr protein kinase
3a	RPS2 RPM1	Arabidopsis Arabidopsis	*Pseudommonas syringae* (avrPto) *P. syringae* (avrRPM1/avrB	CC/NBS/LRR
3b	N L6 RPP5	Tomato Flax Arabidopsis	Tobacco mosaic virus Melampsora lini *Peronospora parasitica*	TIR/NBS/LRR
4	Cf-2, Cf-4, Cf-5, Cf-9	Tomato	*Cladosporium fulvum*	Extracellular LRR
5	Xa21	Rice	*Xanthomonas oryzae*	Extracellular LRR/ Kinase domain

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Chapter 5 ROLE OF PATHOGENESIS RELATED PROTEINS (PRPS) IN PLANT DISEASE MANAGEMENT

Shaik Munnysha, Manisha, Nitisha Gahlot, Ramavath Abhi

Abstract:

Various pathogen attacks on plants from a wide range of pathogenic organisms, such as fungus, oomycetes, bacteria, viruses, and insects, pose a threat to their health and impair agricultural yield. Plants have developed a wide range of defence systems in response to attacks from different pathogenic species. When a pathogen attacks a plant, it activates a number of signal transduction pathways in the host plant and accumulates pathogenesis-related (PR) proteins. Therefore, under conditions of stress such as drought, osmotic, cold, salinity, metal stress, and UV light, PR proteins are typically activated. With regards to plant defence, disease resistance, cell wall rigidification, development, antifungal activity, enzymatic functions (-1,3-glucanase, chitinase, defensins), and stress adaptation, PR proteins serve a variety of roles in plants. There are now seventeen categories for PR proteins. Pathogenesis-related proteins' antimicrobial activity offers useful tools for developing resistance in economically significant plants. The potential use of these proteins in diverse agribiotechnological applications is reflected in their possible future usage. The entire role of PR proteins in plant growth, classification, disease resistance, and their application in genetic engineering to improve disease resistance in agricultural plants

Keywords: Pathogen, PR Proteins, Defence, Resistance, Signal transduction, Genetic engineering

Introduction:

Pathogenesis-related Proteins (PRPs)

Plant proteins that are activated in pathogenic or similar circumstances are known as pathogenesis-associated proteins. Their presence before to infection promotes pathogen resistance. These new proteins show that the plant has developed systemic acquired resistance because they accumulate at their highest levels 7–10 days after infection. (The fact that healthy plants contain modest quantities of these proteins suggests that they may have purposes other than disease resistance.) PRPs build up in the vacuole and in the intercellular gaps. First line of defence against a difficult pathogen is provided by the intercellular PRPs, and in the event that this fails and tissue is damaged, the release of vacuolar PRPs serves as second line of defence by engulfing the pathogen with lytic enzymes that have antifungal, antibacterial, and antiviral activity.

Seventeen families of PRPS have been officially recognized (Van Loon *et al.,* 2006), most of which have enzymatic activities. These are endochitinases, B-1,3 glucanases, proteinases, proteinase inhibitors, lysozymes, ribonuclease, thaumatin-like, defensin, thionin, lipid-transferase proteins, oxaloacetate etc.

Pathogenesis-Related Proteins (PR proteins)

- When pathogen makes entry into cuticle, cell wall and cell membrane, accumulation of several proteins in host during plant pathogenesis situations
- These novel proteins are induced which are collectively referred as PR proteins.
- PPR Proteins initially named as "b" proteins were discovered in tobacco leaves, hypersensitively reacting to TMV.

Characteristics

- Established in all plant organs leaves, stems, roots and flowers.
- Low molecular weight proteins (5-75KDa)
- Stable at low pH (<3)
- Thermostable and highly resistant to protease
- Contain 4 α helices and β strands arranged antiparallel between helices

Notes on PR Proteins

- Accumulate not only in local regions, but also occur systemically
- Associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses
- Found in many plant species belonging to various families
- General role in adaptation to biotic stress conditions
- First identified in tobacco TMV
- Cloned and introduced into host plants – Resistance
- β 1,3- glucanase gene- bean plants- introduced into tobacco plants- Transgenics- Resistance to *Peronospora parasitica* (Downy mildew)
- Bean chitinase- Tobacco plants- Resistance to *Rhizoctonia solani*
- Transgenic rice plants- Rice TLP gene/ rice chitinase gene- Resistance to sheath blight.

Classification of PR Proteins

The anti-microbial properties of PR proteins make this family an appealing target for genetic engineering, which could increase resistance by constitutively expressing genes that are induced by defence. Because of their potential application in creating transgenic crops with increased pathogen resistance and reducing the amount of pesticides needed in agriculture, several PR proteins' antimicrobial properties are of tremendous interest to both the agribiotechnological and pharmaceutical industries.

PR-1 family

The first class of PR proteins to be identified, PR-1 is highly expressed and accounts for 10% of the total protein in infected leaves. Plants produce PR-1 proteins in response to host plant cells recognising the pathogen, which triggers the activation of transduction pathways. The PR-1 group belongs to the superfamily of cysteine-rich proteins, has a molecular weight of 15 to 17 kDa, and exists in both basic and acidic isoforms (van Loon *et al.*, 1999). Vertebrates, angiosperms, insects, yeasts, and other organisms all have the PR-1 proteins. Muskmelon was the first fruit to be linked to an allergy in the PR-1 protein family (Cuc m 3). In many plant species, PR-1 proteins serve as helpful molecular markers for the SAR response and plant disease. The first PR-1 protein to be described is PR-1a from N. tabacum. Since then, a number of Arabidopsis, maize, wheat, tomato, tobacco, barley, rice, and pepper PR-1 proteins have been discovered. The basic PR-1 protein from pepper (CABPR1) was also shown to offer increased disease resistance to the pathogens *Phytophthora nicotianae*, *Ralstonia solanacearum*, and *Pseudomonas syringae* as well as partial tolerance to heavy metal stress in transgenic tobacco plants (Sarowar *et al.,* 2005).

PR-2 family: β-1,3-glucanases

In vitro 1,3-endoglucanase activity is present in the 1,3-glucanase PR-2 proteins. The 1,3-glucanase enzymes are quite prevalent and catalyse the endo-type hydrolytic breakage of 1,3-D-glucosidic links in 1,3-glucans, which weakens the pathogen's cell wall and causes it to die. Plant -1,3-glucanases are acidic and basic isoforms with a molecular mass of 33–36 kDa. There is a large diversity of -1,3-glucanases in bacteria, fungi, and plants. In physiological and developmental processes such pollen germination, flowering, cell differentiation, fertilisation, embryogenesis, fruit ripening, mobilisation of storage reserves, and bud dormancy, 1,3-glucanases are critical players. In reaction to wounding, cold, ozone, UV, and a variety of fungal disease attacks on plants, the -1,3-glucanases are substantially stimulated. The gene for 1,3-glucanase was initially discovered in rice. Similar to tobacco plants, many plant species have several copies of the gene for -1,3-glucanase. 14 multiple copies of the 1,3-glucanase gene have been identified. The plant vacuole contains 33 kDa basic proteins called class I glucanases. Extracellular space contains 36 kDa acidic proteins that belong to classes II and III. Furthermore, it has been demonstrated that transgenic cucumbers expressing the class I rice chitinase gene RCC2 displayed improved disease resistance to the grey mould disease-causing *Botrytis cinerea* (Kishimoto *et al.,* 2002). Recently, the biocontrol fungus Trichoderma virens-10's 1,3-glucanase (Bgn13.1) gene was identified, and when expressed in transgenic *Brassica napus,* it has antifungal action against the phytopathogenic fungus *Sclerotinia sclerotiorum* (Kheiri *et al.,* 2014).

PR-3, PR-4, PR-8, PR-11 family: Chitinases

The hydrolysis of the 1,4-linkage in the chitin's N-acetylglucosamine polymer is facilitated by the hydrolytic enzymes known as chitinases. The molecular weight of plant chitinases ranges from 25 to 40 kDa, and stems, seeds, flowers, and tubers all naturally contain both acidic and basic isoforms. Chitinases have lysozymal activity and are present in plants in small amounts, but they are robustly and consistently up-regulated by a variety of biotic and abiotic stress situations. The transgenic plants' increased resistance to pathogen attack is a result of overexpressing chitinases. The four PR families that make up chitinases are PR-3, PR-4, PR-8, and PR-11. Chitinases contribute to plant defence by preventing or delaying the formation of hyphae that infiltrate the intercellular space or by releasing fungus elicitors. Chitinase are classified into two categories endochitinases (EC 3.2.1.1.4) and exochitinases (EC 3.2.1.14) with respect to their hydrolytic sites. Endochitinases cleaves chitin at internal bonds producing soluble, low molecular mass multimers of N-acetyl glucosamine such as chitotrose, chitotetraose and di-acetylchitobios. The endochitinases includes PR-3, PR-4, PR-8 and PR-11 families. Exochitinases cleave chitin by releasing diacetylchitobiose without producing N-acetyl glucosamine or oligomers.

Chitinases are widely present in different tissues with multiple isoforms and gene clusters in both monocots and dicots plant species. Further on the basis of structure, specificity, catalatic mechanism and inhibitor sensitivity the plant chitinases are classified into seven classes I-VII. In tobacco Class I, V and class VI chitinases posses anti-fungal activity in vitro against Fusarium solani, Alternaria radicina and Trichoderma viride. Recently Prasad *et al.* (2013) demonstrated that overexpression of a chitinase gene from rice (Rchit) in transgenic peanut plants confers enhanced resistance against soil borne pathogens *Phaeoisariopsis personata* and *Puccinia arachidis* respectively.

PR-5 family: Thaumatins

Thaumatin-like proteins (TLPs), which are PR-5 protein members, share a high degree of sequence identity with thaumatins, a protein with a sweet flavour that has been isolated from the West African plant *Thaumatococcus daniellii*. The PR-5 family of proteins performs a variety of roles in plant disease resistance, including antifungal activity, antifreezing activity, and tolerance to osmotic stress. In Arabidopsis, about 24 PR-5 genes have been discovered. TLP, osmotin, osmotin-like proteins (OLP), and zeamatin are members of the PR5 family. TLPs have a molecular mass between 20 and 26 kDa and contain about 200 residues with 16 conserved cysteine residues that form eight disulphide linkages. The PR5 family is divided into basic and acidic members based on their isoelectric points. The basic member proteins include an extra C-terminal extension that targets the vacuole, while the acidic members are distinguished by the inclusion of an N-terminal signal peptide. The PR-5 family represents pollen or food derived

allergens that are present in various plant species. Several pollen allergens have been detected in Eastern red cedar (Jun v 3), Japanese cedar (Cry j 1), mountain cedar (Jun a 3), Arizona cypress (Cup a 3) and food allergens include apple (Mal d 2), sweet cherry (Pru av 2), bell pepper (Cap a 1), kiwi (Act d 2), peach (Pru p 2) and banana (Musa 4). The thaumatin like proteins posseses antifungal activity by inhibiting hyphal growth, spore lysis and/or reduction in spore germination or viability of germinated spores. It is demonstrated that in vitro overexpression of PR-5 proteins in potato delayed development of disease symptoms of *P. infestans* which is the cause of late blight disease of potato (Liu *et al.,* 1993). Osmotin is a 24-kDa, fundamental pathogenesis-related protein that functions as a fungus's defence mechanism when it comes to a range of pathogenic fungi. *Phytophthora infestans, Fusarium oxysporum, Altermaria solani, Botrytis cinerea, Fusarium oxysponun, Verticillium akhliae, P. nicotianae,* and *Cercospora beticola* are only a few of the phytopathogenic fungi that osmotin inhibits in vitro spore germination and hyphal development against. A 25-kDa TLP called linusitin that was isolated from flax seeds has antifungal properties against *Alternaria alternata*. Zeamatin, a 22 kDa antifungal protein derived from maize, also contributes significantly to plant defence. Osmotin and Zeamatin, PR-5 proteins from maize, have antifungal properties because they permeabilize fungal membranes. According to research by Wang et al. (2011), *Verticillium dahliae, Fusarium oxysporum, Rhizoctonia solani, Botrytis cinerea, and Valsa mali* are among the fungi that are strongly inhibited by thaumatin-like proteins (CkTLP) that were isolated from the seeds of the dessert plant *Cynanchum komarovii*. Additionally, over-expression of the antifungal proteins CkChn134 and CkTLP from *C. komarovii* seeds increases the plant's resistance to V. dahliae in transgenic Arabidopsis thaliana (Wang *et al.,* 2011). Furthermore, Zamani *et al.* (2012) showed that Canola (*Brassica napus* L.) expressed the thaumatin-like tlp gene that was identified from cereal rye (*Secale cereal* L.), which imparts antifungal activity and increases resistance to the fungus *S. Sclerotiorum* that causes stem rot disease. Recent research has shown that overexpression

PR-6 family: Protease inhibitors

Small proteins known as proteinase inhibitors (PIs) from the PR-6 family function as protease's natural adversaries. Inhibiting the activity of proteases, proteinase inhibitors are primarily found in storage tissues (tubers and seeds) and aerial sections of plants. Plant PI proteins have a size range of 4 to 85 kDa and contain a lot of cysteine residues, which help to build disulfide bridges. In response to numerous stresses, including as pathogen and insect attacks, wounding, and various environmental challenges, PIs, which are involved in plant defence, are activated. Cowpea trypsin inhibitors have broad-spectrum efficacy against a variety of nematodes, including *Globodera tabaccum, G. pallida*, and *Meloidogyne incognita* (CpTi). Many plants have PIs that serve significant defense-related activities, such as potato and tomato proteinase, cowpea trypsin inhibitor, and others. As many plant PIs potently inhibit the growth of

pathogens therefore used as excellent candidates for the development of novel antimicrobial compounds. Additionally, protease inhibitors prevent microbial development through an antifeedant mechanism. Based on the active amino acid in their "reaction centre" and the four classes of proteinases they block, the PIs families are divided into serine, cysteine, aspartic, and metallo-proteases (Koiwa *et al.,* 1997). Serine PIs are one of several forms of plant PIs that are common across the plant kingdom. However, since insects like *Helicoverpa armigera* and *Spodoptera litura* require serine proteinases as important digesting enzymes, serine PIs are used to develop insect resistance in transgenic plants. In plants, animals, and microbes, cysteine proteinase inhibitors (also known as cystatins or cysteine PIs) are widely dispersed and prevent cysteine proteases from working. Oryzacystatin is the first cysteine PI that has been isolated and is well-characterized (OC-1). The metallocarboxypeptidase inhibitors are tiny peptide inhibitors having a molecular mass of less than 4.2 kDa and 38–39 amino acid residues. During development, this inhibitor builds up in a variety of tissues, including the potato tuber and leaf. In addition, the potato's response to injury results in the accumulation of the potato inhibitor I and II families of serine PIs, which are potent inhibitors of a variety of pathogens' carboxypeptidases. Trypsin, chymotrypsin, and the aspartyl protease cathepsin D are all inhibited by the 27 kDa cathepsin D inhibitor, also known as an aspartyl PI. To create transgenic plants that are insect-resistant, many plant species have been exploited to overexpress the various protinase inhibitor families. It has been demonstrated that the overexpression of serine proteinase inhibitor gene (SaPIN2a) from *Solanum americanum* in transgenic tobacco shows enhanced resistance to lepidopteran insects *Helicoverpa armigera* and *Spodoptera litura* which causes considerable economic loss in cotton, tobacco, sunflower, corn, pepper and tomato plants (Luo *et al.,* 2009).

PR-7 family: Proteinase (Endoproteinase)

Proteinases and peptidases, also known as proteases, are crucial for the regulation of many biological activities, including growth, development, photosynthesis, and the induction of defence mechanisms against herbivores, insects, and nematodes. In addition to releasing peptide-based toxins or activating enzymes from their precursor proteins, proteases directly break down proteins from the pathogen. Over 800 proteases are encoded in the Arabidopsis genome. Protease inhibitors reduce the activity of proteases. Endopeptidases and exopeptidases are the two types of proteases. Trypsin, chymotrypsin, pepsin, papain, and elastase are examples of endopeptidases that hydrolyze internal peptide bonds in polypeptide chains, whereas exopeptidases break down terminal residues, such as aminopeptidases and carboxypeptidase. Due to the uncharacterized substrates in plants, many plant proteases that are released into the apoplast and play a role in defence are poorly understood. Proteases are further grouped according to their active sites and mechanisms of action into five major classes: (i) serine proteases (ii) aspartic proteases (iii)

cysteine proteases (iv) threonine and (v) metalloproteases. Serine proteases are a largest class of proteolytic enzymes which cleave peptide bond in proteins where the active site Ser acts as a nucleophile. Serine proteases are involved in a number of vital physiological processes that control how plants develop and mount defences. During the proteolytic process, cysteine proteases utilise a catalytic cyst as a nucleophile. Cysteine proteases control the development of pollen or embryos, flowering period, inflorescence architecture, and epidermal cell fate. Cysteine proteases are crucial in the localised PCD that occurs in response to pathogen infection. Insecticidal properties can be found in cysteine proteases. The plant cysteine proteases obtained from *Arabidopsis* (AtCP2) and *Gossypium hirsutum* (GhCP1) have recently been shown to be resistant against insect cotton bollworm larvae (Mao *et al.,* 2013). The second-largest protease class is known as aspartic proteases (AP), which include two aspartic residues that serve as the nucleophile during proteolysis. Two aspartic acid residues in the conserved (Asp-Thr/Ser-Gly) pattern, which is responsible for catalytic activity, are present in APs.

PR-9 family: Peroxidases

Peroxidases are a common type of oxidoreductases that catalyse the oxidation of several organic and inorganic substances as well as the reduction of hydrogen peroxide. The primary enzymes involved in a wide range of physiological activities include auxin catabolism, hormone signalling, wound healing, lignification, suberization, and reaction to stress in plants. Plant peroxidases are heme-containing enzymes with a molecular weight of 32–42 kDa that catalyse the oxidation of substrates such as phenol and its derivatives by hydrogen peroxide. Peroxidases have a variety of roles in the host plant's defences against various necrotrophic or biotrophic diseases, including their involvement in the polymerization of lignin precursors. Important resistance gene markers for disease resistance are frequently employed with peroxidases. Many plant species have had their peroxidases described and purified. The genome of *Arabidopsis thaliana* is said to have 130 peroxidases. Pathogens and other stresses, such as drought, high light levels, salinity, low temperatures, metal stress, and UV light, cause peroxidase expression. According to their structural characteristics, peroxidases can be roughly divided into three types. Chloroplasts, mitochondria, peroxysomes, and cytosol are the intracellular peroxidases known as class I peroxidases. Yeast cytochrome c peroxidase, ascorbate peroxidase, and cytosol of higher plants are examples of class I peroxidases. Monomeric glycoproteins called Class II peroxidases break down lignin, which is made up of secretory fungal peroxidases. Ligninases and manganese-dependent peroxidases are included in class II. Four conserved disulphide bridges and two conserved calcium binding sites make up the structure of class II peroxidases. Class III are glycoprotein secretory plant peroxidases located in vacuoles and cell walls which play role in various functions like defence responses towards wounding, indole-3-acetic acid, oxidation of toxic compounds, ethylene biosynthesis (Passardi *et al.*, 2005). Two conserved calcium ions,

four conserved disulphide bridges, and one N-terminal signal peptide are all present in class III peroxidases. The Populus peroxisomal ascorbate peroxidase (PpAPX) gene is overexpressed in tobacco plants, which improves their ability to withstand stress. According to research by Sarowar *et al*. (2005), tobacco plants that overexpress the Capsicum annuum ascorbate peroxidase-like 1 gene (CAPOA1) have greater tolerance for oxidative stress as well as enhanced resistance to the oomycete pathogen Phytophthora nicotianae. Anionic peroxidase enzyme was overproduced in transgenic tobacco, tomatoes, and sweet gum, and these plants showed increased resistance to insect assault. Recent research has shown that tobacco transgenic plants overexpressing the *Salicornia brachiata* ascorbate peroxidase SbpAPX gene can tolerate salt and drought stress (Singh *et al.,* 2013).

PR-10 family: Ribonuclease-like protein

Small, acidic proteins in the PR-10 family have molecular weights between 15 and 18 kDa. Tree pollen allergies and the main food allergens of celery or apples are under the PR-10 family. Due to their structural resemblance to ginseng ribonuclease, PR-10 proteins are categorised as ribonuclease-like PR proteins. The RNase and ligand-binding properties of PR-10 proteins defend plants by inducing programmed cell death around infection sites or by directly attacking pathogens. The PR-10 proteins are crucial for the growth, development, and defence mechanisms of plants against a variety of biotic and environmental challenges, including UV radiation, high salt, low and high temperatures, wounding, and dehydration. Members of the PR-10 protein family, which have been closely researched in birch (Bet v 1) homologues, also function as pollen allergens. Several Bet v 1 homologus have been characterized and isolated in apple (Mal d1), sweet cherry (Pru av 1), apricot (Pru ar 1), celery (Api g 1), peach (Pru p 1), pear (Pyr c 1), carrot (Dau c 1), parsley (pcPR-1 and pcPR-2), potato (pSTH-2 and pSTH-21), hazelnuts (Cor a 1) and chestnuts (Cas s 1) (Hoffmann Sommergruber 2002). Both dicots and monocots, such as the bean, soybean, asparagus, sorghum, barley, rice, potato, apple, and *Medicago sativa*, have been isolated from the PR-10 family. High quantities of PR10 proteins have been found in birch pollen, hornbean, apple, celery, pear, and soybean as food or pollen allergens. The *P. capsici* pathogen is prevented from growing by the recombinant CaPR-10 protein from *Capsicum annuum,* which also has ribonucleolytic activity against the RNA of the tobacco mosaic virus. The Oxalis tuberosa's unique tuber storage PR-10 protein Ocatin has antibacterial and antifungal properties. Recently, Xie *et al.* (2013) demonstrated that overexpression of PR10 family gene ARAhPR10 play an important role in *Arachis hypogaea* seed resistance to *Aspergillus flavus.*

PR-12 family: Defensin

Plant defensins are positively charged, 45–54 amino acids long, low in molecular mass (3–5 kda), and rich in basic cysteine residues that form disulfide bridges. Defensin has a wide

range of powerful antifungal, antibacterial, proteinase-inhibitory, and insect amylase-inhibitory activities. The first defensins were isolated from the endosperm of wheat and barley. Later, it was discovered that the *A. thaliana* genome encodes more than 300 peptides similar to defensins. In addition, plants' responses to biotic and abiotic stress result in highly expressed or upregulated plant defensin genes. Four categories are used to group defensins. Group I defensins are referred to as morphogenic defensins because they alter the morphology of fungi that are vulnerable to them. Group II proteins are non-morphogenic and prevent fungal growth without altering morphology. Group III inhibits -amylases in vitro but has little antifungal action. Unique antifungal specificity is found in Group IV. Plant defensin has been found to exhibit antifungal properties in a variety of plants, including pea, tobacco, radish, and arabidopis. *Raphanus sativus* yields two plant defensins with excellent characterization that have both in vivo and in vitro antifungal action (RsAFP1 and RsAFP2). Strong fungal pathogen resistance is provided by transgenic plants overexpressing defensins, which also offer broad-spectrum pathogen resistance. Increased resistance to *Verticillium dahliae* is provided by the plant defensin AlfAFP, which was isolated from the seeds of *Medicago sativa.*

PR-13 family: Thionin

Thionins are a class of basic, low-molecular-weight polypeptides with a molecular mass of about 5 kDa that are tiny, cysteine-rich, and stabilised by conserved cysteine residues that form 3–4 disulfide-linked bonds from 44–48 amino acid residues. Thionin functions as a plant defence protein and has antibacterial, antifungal, and the capacity to block insect -amylases and proteinases. Numerous thionins have been found and isolated in various plant species up to this point. Thionins cause the creation of open pores in the cell membranes of phytopathogens, which releases calcium and potassium ions from the cell. Five categories can be used to categorise thionins (I-V) Purothionins, type I thionins, are extracted from the endosperm of wheat and barley (hordothionins), which contains eight cysteine residues among its 45 highly basic amino acid residues. Eight cysteine residues, which form four disulfide bridges at the same places as those of type I, are present in type II thionins, which are 46–47 amino acids long. The core disulfide loop of the 46 residues that make up type III thionins has three conserved disulfide bridges. The viscotoxins and phoratoxins from mistletoes isolated from the leaves and stems of *Dendrophthora clavata, Phoradendron tomentosum, Phoradendron liga,* and *Viscum album* are included in the type III thionins. The type IV consists of 46 neutrally charged amino acid units and three disulfide linkages. The crambins that were isolated from the Abyssinian cabbage relate to the type IV thionins. Similar to type IV, type V thionins, which have been found in the endosperm of wheat and *Aegilops species*, are also neutral.

PR-14 family: Lipid transfer proteins

The ability to bind lipids and other hydrophobic compounds is possessed by lipid-transfer proteins (LTP), which are tiny (9–10 kDa), basic, soluble, and ubiquitous proteins. Four disulphide bonds involving eight cystine residues, which create a tunnel-like hydrophobic chamber for ligand binding, stabilise the alpha helical shape of LTP. LTP has a variety of functions in plant development and defence, including cutin production, -oxidation, somatic embryogenesis allergies, pollen adherence, signalling, and phytopathogen defence. In reaction to environmental conditions like drought, cold, and excessive salinity, LTPs express themselves. Additionally, a number of signalling chemicals from the signalling pathway, including ethylene, salicylic acid, methyl jasmonate, and abscisic acid, are responsible for the development of LTP. The plant's aerial and vascular tissues, which are exposed to pathogens at a high level, have a large concentration of LTPs. A small multigene family from different plant species that encodes LTPs in plants plays a crucial role in a number of processes, including cuticle biosynthesis and embryogenesis. LTPs are divided into the LTP1 and LTP2 families. LTP1 have 10 kDa or less molecular weights and are basic. Eight conserved cysteine residues in LTP1 help to stabilise the protein's structure by forming four disulfide bridges among its 90–95 amino acid residues. The LTP2 family has molecules with average molecular weights of 7 kDa and 70 amino acids.

PR-14 family members are most important allergens detected in many plant species like peach (Pru p 3), apple (Mal d 3), apricot (Pru ar 3), cherry (Pru av 3), plum (Pru d 3), hazelnut (Cor a 8), chestnut (Cas s 8), maize (Zea m 14), barley (Hor v 14), Parietaris judaica (Par j 1), grape (Vit v l), asparagus (Aspa o 1), *H. brasiliensis* latex (Hev b 12), mugwort (Art v 3), ambrosia (Amb a 6). LTPs in radish, maize, and grape have been shown to have in vitro antibacterial properties against different fungi and bacteria. *Phytophthora nicotianae*, an oomycete pathogen, and *Pseudomonas syringae pv. tabaci*, an oomycete pathogen, were both more susceptible to tobacco plants with constitutive expression of the CALTPI and CALTPII genes. Recently, Zhu *et al.* (2012) showed that transgenic *Triticum aestivum* plants overexpressing the lipid transfer protein gene TaLTP5 exhibit increased resistance to the wheat diseases common root rot and fusarium head blight, respectively, caused by *Cochliobolus sativus* and *Fusarium graminearum.*

PR-15, 16 family: Oxalate oxidase

Oxalate oxidases (OXO), which are found in plants in low concentrations, are essential for biotic and abiotic stress defence. Oxalic acid and oxygen are aerobically oxidised by the enzyme, producing CO_2 and H_2O_2. H_2O_2 also initiates a signal transduction cascade that stimulates plant defence systems and results in the production of phytoalexins and proteins involved in disease (Mittler, 2002). With regard to germination, fruit ripening, floral induction, seed development, embryogenesis, nodulation generation of H_2O_2, and nitrogen fixation, the

oxalate oxidase plays a variety of physiological and defensive activities. *Hordeum vulgare* and *Triticum aestivum* were the first plants where oxalate oxidase was discovered and defined. OXO exists in two forms in nature: soluble and membrane-bound. The best-characterized enzyme is wheat germ oxalate oxidases, which have high heat and protease resistance. As a result, germin have been extracted from both dicots and monocot species and are employed as a marker for early plant growth. Oxalate oxidase and oxalate oxidase-like proteins of six different types have been identified in barley. Increased resistance to OA-producing diseases such *Sclerotinia sclerotiorum, Cristulariella pyramidalis*, and *Septoria musiva* is a benefit of OXO's constitutive expression. Millimolar quantities of oxalic acid are produced and secreted by the pathogen into the infected host tissues. By destroying the OA created by the Sclerotinia toxin, which lessens the pathogen's harm to plant tissues, the OXO considerably reduces the Sclerotinia illness and creates the defense-inducing molecule H_2O_2. *Colocasia esculenta* (Taro) modified with the oxalate oxidase gene gf2.8 from wheat recently demonstrated enhanced resistance to Taro, according to Xia *et al*.

PR proteins families

Family	Properties	Type member
PR-1	Unknown	Tobacco PR-1a
PR-2	β-1,3- glucanase	Tobacco PR-2
PR-3	Chitinase type I, II, IV, V, VI and VII	Tobacco P, Q
PR-4	Chitinase I, II	Tobacco ‘R’
PR-5	Thaumatin-like protein	Tobacco S
PR-6	Proteinase- inhibitor	Tomato Inhibitor I
PR-7	Endoproteinase	Tomato P_{69}
PR-8	Chitinase type III	Cucumber Chitinase
PR-9	Peroxidase	Tobacco ‘lignin-forming peroxidase’
PR-10	Ribonuclease- like protein	Parsley ‘PR1’
PR-11	Chitinase, type I	Tobacco Class V Chitinase
PR-12	Defensin	Radish Rs-AFP3
PR-13	Thionin	Arabidopsis THI2.1
PR-14	Lipid- transfer protein	Barley LTP4
PR-15	Oxalate oxidase	Barley
PR-16	Oxalate oxidase like	Barley OX OLP
PR-17	Unknown	Tobacco PRp27

PR-17 family: Unknown

The PR17 proteins are crucial for plant defence against pathogens, although their precise molecular roles are still unknown. Additionally, it has been shown that wheat (WCI-5) overexpressing the PR17 protein gives resistance to the powdery mildew fungus *B. graminis f.sp. tritici* (Schweizer *et al.,* 1999). NtPRp27 is produced in tobacco in response to mechanical trauma and infection with the tobacco mosaic virus (Okushima *et al.,* 2000). Furthermore, it was shown that the barley encoded proteins HvPR-17a and HvPR-17b are members of this family. These proteins are monomeric polypeptides with molecular weights of 26 and 24 kDa respectively (Christensen *et al.,* 2002). These proteins are apoplastic which accumulates in the barley mesophyll apoplast and in leaf epidermis when attack with powdery mildew fungi *Blumeria graminis* f.sp. *hordei*. The HvPR-17a and HvPR17b proteins are present at different level in the plant.

PR Proteins in brief

Stress proteins might include PR-proteins. Plants produce specialised proteins, such as heat-shock proteins, drought-stress proteins, or freeze proteins in response to environmental conditions. The induction of partially comparable protein sets by abscisic acid suggests that acclimation is a hormone-controlled process. PRPs are thus stress proteins that are created specifically in response to necrotizing infections by viruses, viroids, fungi, and bacteria. They play a role in the development of acquired resistance to subsequent infections. But unlike the majority of stress proteins, the PRPs accumulate in considerably higher amounts and are quickly found on gels by protein staining. Up to 1% of all soluble proteins may be present in the concentration. Van Loon (2006) found that flower tissues naturally contain a large number of defense-related proteins.

Conclusion:

Proteins associated with pathogenesis offer strong defences against a variety of pathogenic and phytopathogenic bacteria. A deeper understanding of plant development and disease resistance is provided by the growing body of information on PR proteins. The PR genes are regarded as "stress-inducible" proteins with a variety of uses in crop improvement genetic engineering. In the agricultural industry, PR proteins' antibacterial properties are utilised to develop genetically modified plants with improved field resistance. Additionally, the combination of different PR proteins creates new opportunities for genetic engineering and sheds light on the pathogen defence mechanism with enhanced disease resistance for the creation of novel and improved crop types. In light of increased plant disease resistance to numerous pathogens and less significant pesticide use, pathogenesis-related proteins may contribute to an improvement in crop yield.

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Chapter 6 ROLE OF PHYTOALEXINS IN PLANT DEFENSE MECHANISM

Ramavath Abhi, Nitisha Gahlot, Manisha, Shaik Munnysha

Introduction:

Low molecular weight, antimicrobial compounds that are synthesized and accumulated in plants after exposure to microorganisms. Their formation and accumulation is often associated with hypersensitive response, although only living cells surrounding the dead cells synthesize phytoalexins. While HR occurs in minuets, phytoalexin accumulation takes hours.

Phytoalexins can develop resistance to the pathogen's future infections. Cell wall fragments are a biotic elicitor of phytoalexin production, as are abiotic elicitors such heavy metals and ultraviolet light. Phytoalexins are non-persistent and non-translocated.

In more than 100 plant species, there are over 350 phytoalexins. They belong to various classes, including terpenoids, glycosteroids, and alkaloids, and differ greatly in terms of their structure and chemical make-up. Similar in structure are the phytoalexins produced by related plant species. Many plants produce multiples, exposing the invader to a diverse mixture of poisonous compounds

The variety of phytoalexins reveals their mode of survival. A plant's chances of developing disease resistance are improved if it produces phytoalexins that are distinct from those made by its neighbours. Plants produce antibiotics by a metabolic process that is either biotically or chemically or environmentally stimulated.

Phytoalexins synthesis when challenged with pathogens

Crop	**Pathogen**	**Phytoalexin**
Bean	*Colletotrichum lindemuthianum*	Phaseollin, Phaseollidin, Kievitone
Tomato	*Alternaria solani*	Rishitin
Soybean	*Phytophthora megasperma*	Glyceollin
Alfalfa	*C. lindemuthianum*	Medicarpin
Peas	*Nectria*	Pisatin
Grapes	*Uncinula necator*	Stilbenes (Stilbene synthase)
Castor	*Rhizopus*	Casbene (Casbene synthatase)
Rice	*Pyricularia grisea* and *Fusarium moniliformae*	Momilactone A and B

Chapter 7 GENETIC ENGINEERING FOR PLANT DISEASE RESISTANCE

Nitisha Gahlot, Shaik Munnysha, Ramavath Abhi and Manisha

Introduction:

The direct altering of an organism's genome through biotechnology is referred to as genetic engineering, sometimes known as genetic modification. By first extracting and copying the relevant genetic material using molecular cloning techniques to produce a DNA sequence, or by synthesising the DNA construct, and then introducing it into the host organism, new DNA can be inserted into the host genome.

One of the most significant plant diseases is plant viruses. There are several repercussions of virus infestation on agricultural regions, ranging from decreased crop quality to full plant destruction. Because of complex interactions with the machinery of plant cells, certain viruses can colonise a variety of hosts, whilst others can only infect a single specified species. New virus kinds originate as a result of mutations in the viral genome, while others are pushed out. For agriculture, the development of pathogenic strains is particularly crucial. To execute the proper control measures, disease management techniques require in-depth knowledge of virus infection and its impact on host plants.

To reduce the virus's ability to spread throughout the plant and/or plantation, many methods have been employed. A virus can spread by vectors, infected seed, or common agricultural procedures used during culture. Certain viruses may appear less frequently if certified seeds are used. Additionally, vector population management and the adoption of clean agricultural techniques can significantly reduce the virus's ability to propagate. In general, viral distribution into a viable plant cell is made possible by disruption to the barrier made up of the cell wall and plasma membrane, a process known as inoculation. Virus particles will then reproduce and propagate throughout the host through plasmodesmata and vascular bundles if a favourable interaction between the virus and the plant cell takes place? These processes' strength will vary depending on the relationship between the virus and the plant host. The set of plant resistance responses aims to reduce virus replication. In some cases, breeding cultivars with elevated resistance levels represents a viable strategy to reduce the virus-induced crop loss.

Life cycle of a plant virus:

A brief description of the usual positive-sense, single-stranded RNA viral life cycle is given. Plant viruses can enter plant cells passively by mechanical methods or through vectors. The virion becomes uncoated upon entrance, releasing the viral RNA. The viral RNA is subsequently translated from 5' to 3', initially by the RNA-dependent RNA polymerase to

manufacture the gene products needed for the replication of the RNA and possibly utilising both viral and host-encoded components. For the expression of the numerous viral genes, plant viruses use a variety of methods, either separately or in combination, including read through, translational frame shift, polyprotein processing, creation of sub-genomic RNA, or divided genomes.

Additional non-structural genes, coding for movement protein, pro-tease, and helper component/acquisition factor for vector-transmission, are synthesised at different stages in the viral life cycle in addition to the structural genes, such as the coat protein needed for packaging of progeny viral RNA. The coat-protein subunits wrap progeny viral RNA into virions in the last stage. We are learning more and more about the molecular underpinnings of viral gene control, expression, virus movement, and transmission.

When one or more of the viral life cycle events are inhibited, genetically engineered cross protection may follow. Coat-protein-mediated protection, inhibition by antisense RNA, non-structural gene-mediated protection (such as replicase, movement protein, and viral protease), satellite RNA, virus-specific antibody genes, defective, interfering RNA, and genes of non-viral origin are a few strategies used to accomplish this goal.

The idea of engineering resistance at the gene level by inserting specific, precisely specified sequences of viral origin has been explored by scientists explore the availability of genetic information on plant viral genomes and accomplishments in plant transformation and regeneration technology. This is often done by introducing the viral sequence(s) through transformation mediated by *Agrobacterium tumifaciens*. Direct DNA uptake by protoplasts and biolistic bombardment of plant tissue are other successful methods for altering the genetic makeup of plants.

How do the plants defend themselves against viruses?

Viruses promote the infection of susceptible hosts by various strategies that involve well-documented modifications in host plant cells to enhance infection. Initially, replication complexes produce abundant amounts of viral genome followed by the formation new virus particles. At this stage, some viruses are able to suppress plant gene silencing strategies. Interference with cell cycle regulation and cell-to-cell trafficking as well as loss of photosynthetic activity may also occur. Virus spread within the plant body exploits cell-to cell and long-distance pathways. Plasmodesmata are used to allow the virus particles to move from the inoculation site to neighbouring cells. Since plants control trafficking between the cells mainly by alteration of the plasmodesmata diameter, some viruses synthesize specialized movement proteins that overcome this barrier and enhance the pore diameter.

In this way, the majority of viruses are loaded into phloem arteries and delivered to various plant organs along with the photoassimilates. As evidenced by the Cowpea severe

mosaic comovirus in soybean, many particles are now ready to start a new life cycle by spreading to another appropriate plant, for instance through an insect vector. However, the host is not unresponsive throughout these procedures. Plants can fend off infection if their general defence mechanisms are engaged or if they have resistance genes that produce anti-invading virus proteins. The implementation of the necessary preventative measures benefits from extensive understanding of such responses, which can be either generic or specialised.

Resistance genes that are dominant and recessive provide the foundation of the natural plant immune system. According to this paradigm, pathogen avirulence (Avr) genes interact with plant dominant resistance genes (R) in an allele-specific manner. Hypersensitive response (HR), a type of localised programmed cell death, is frequently noticed in this kind of encounter. A basal response imparted by the recessive resistance genes can also happen, so reducing the extent of invasion even though it does not stop the pathogen from invading the host. Generally speaking, based on their anticipated protein structures, all known dominant R genes have been divided into eight classes. Only nine R genes have been isolated and sequenced: N, Rx1, Rx2, Sw5, Tm2, HRT, RTM1, RTM2 and RCY1. The majority of the proteins that are encoded by these genes have putative leucine-zipper (LZ) or other coiled-coil (CC) amino acid sequences at their N-termini, a nucleotide-binding site (NBS) sequence in the middle, and leucine-rich repeats (LRR) of different lengths at their C-termini. To now, the NBS-LRR class comprises all R genes that provide viral resistance. The R genes that confer HR can identify genomic regions, motility proteins, coat proteins (CP), and viral RNA polymerase subunits as avirulence factors.

A mechanism to explain the genetics of Avr-R genes disease resistance would be that R gene products serve as the direct receptors for pathogen encoded Avr proteins. An alternative mechanism would be that R proteins would form complexes that would recognize the pathogen molecules in the initial invasion stages. Binding of pathogen molecules would lead to a Less is known about the plant responses controlled by the recessive resistance genes. The majority of the proteins produced by these genes contain putative leucine-zipper (LZ) or other coiled-coil (CC) amino acid sequences at their N-termini, a central nucleotide-binding site (NBS) sequence, and C-terminal leucine-rich repeats (LRR) of different lengths. All R genes that give viral resistance as of right now are NBS-LRR genes. As avirulence factors, viral RNA polymerase subunits, motility proteins, coat proteins (CP), and genomic segments can all be recognised by R genes leads to HR.

Molecular strategies to protect crop plants from virus infection:

Transgenics with pathogen-derived resistance

Transgenics that are resistant to an infectious virus have been created in a number of crops by genetically inserting a viral genome sequence into the desired crop. Many crops have been transformed into virus-resistant varieties by inserting either viral CP or replicase gene

expressing sequences. Resistance produced by CP is commonly referred to as CPMR. Research on replicase-mediated resistance has been conducted in a number of labs, and in the majority of these studies, it was discovered that the resistance was caused by a post-transcriptional gene silencing (PTGS) response that occurs naturally in plants. Because of the essential nature of the viral movement protein for intercellular movement of plant viruses, movement problem sequence has also been used for achieving viral resistance. Other pathogen-derived approaches described in the literature, include the use of satellite RNA and defective-interfering viral genomic components.

1. Coat-Protein-Mediated Protection (CPMP)

The ability of tobacco plants that had been stably transformed with the TMV coat-protein (CP) gene to resist future TMV inoculation was originally shown by Beachy and colleagues. They created a chi-mer gene using the TMV CP gene, flanked by the 35s CaMV promoter and transcription termination and ployadenylation signals from the A. tumefaciens nopaline synthase gene. Kanamycin was used as the selectable marker after co-cultivation of the Agrobacterium containing the chimeric plasmid with tobacco leaf discs. Southern, Northern, and Western blot studies verified the CP gene's steady integration and expression into the tobacco genome.

The transgenic tobacco lines confirmed as expressing CP were challenge-inoculated with a purified preparation of TMV by mechanical inoculation and the time course of disease development was then monitored. The plants either showed no symptoms or delayed disease development compared with controls (untransformed plants and plants that had only been transformed with the transformation vector without the CP). Resistance to virus infection was manifested at three levels: there were fewer sites for infection; the virus accumulated at a lower level; and there was a delay in symptom development, the rate of systemic spread of the virus being slower in plants expressing the CP than in the controls.

Powell-Abel *et al.* (1986) also noted some limitations to CPMP. Whereas challenge-inoculation using a purified virus preparation elicited a resistance response, the resistance broke down when viral RNA was used as an inoculum (although the latter is not a natural method of inoculation in an agricultural situation). Resistance was less effective at higher inoculum concentrations although a lOO- to lOOO- fold increase in the concentration was required to induce disease symptoms in CP expressers comparable with those in the control plants. Higher temperatures also appear to reduce the level of protection conferred by CP. The mechanism of action by which the CP confers resistance is not clear. While the production of CP is essential for protection against TMV, just the mRNA of the CP was found sufficient to confer protection against other viruses. It seems that no single mechanism is applicable for all the viruses, and different types of mechanisms may be operating at different levels during each virus infection.

Strategies to create Coat Protein-Mediated Resistance

Construction of chemeric genes to encode coat proteins:

First step:

- Isolation and generation of cloned cDNA of coat protein cistron
- Elimination or alteration of nucleotide sequence that (may) affect stability of translation of the transcript
- Placing the initiation codon AUG (Initiator) within the context of the consensus sequence to assure efficient translational initiation
- Some plant viral CP genes with non-translated regions at both the 5' and 3' ends of the coding regions, thus reduce possibility of the base paring by keeping these sequences short and generally low in potential G:C base pairs

Second step:

- Selection of an appropriate transcriptional promoter as plant RNA viruses do not contain promoters capable of transcription in plant chromosome
- Most effective promoter: P35S from cauliflower mosaic virus

Third step:

- Construction of 3' sequence that confers termination and ploydenylation (poly a) to the transcript

Introduction of chemeric gene and regeneration of plants

- DNA copy of the coat protein gene of the virus inserted into genome of susceptible plant through Ti plasmid of the *Agrobacterium tumefaciens*

Possible mechanism:

- Resistance due to early uncoating event
- Restriction in the re-encapsidation of un-coated RNA of challenge virus by endogenous coat protein
- Thus transgenetically expressed coat protein blocks the disassembly reaction towards assembly

The use of CPMP against viruses in several economically important crop plants is summarized below.

Alfalfa: Alfalfa mosaic virus (AlMV) cannot infect transgenic tobacco and tomato plants that are expressing the CP gene of AlMV. In temperate climates around the world, alfalfa is a significant feed crop, and AlMV significantly reduces alfalfa yield. Transgenic alfalfa plants expressing the CP gene were demonstrated to be resistant to challenge inoculation by AIMV by Hill et al. (1991). While the control plants exhibited disease signs, the CP-expressing lines remained symptomless.

Barley: Fertile barley plants were successfully transformed and regenerated, according to Wan and Lemaux (1994). Particle bombardment of immature zygotic embryos, young calluses, and embryos produced from microspores was used to achieve this.

The ability of transgenic lines of cv Golden Promise to confer resistance to BYDV infection is being tested. These lines express the CP gene of the P-PAV isolate of the serious cereal virus disease known as barley yellow dwarf virus (BYDV). The viruliferous aphid vector was used to test the CP-expressing transgenic lines for resistance. Plants were assayed by ELISA for virus multiplication and several lines showed significant resistance, expressed as lower virus titres than for untransformed controls.

Cucumber: Cucumber Mosaic Virus (CMV) causes serious yield losses in a diverse group of crop plants. The CP gene of CMV, when expressed in tobacco, was shown to confer resistance when the plants were challenge-inoculated with CMV extended these observa- tions to cucumber and evaluated transgenic cucumber plants (cv Poinsett76) expressing the CP gene of CMV in a 3- year field trial under natural field conditions with natural aphid populations.

The degree of resistance displayed by the transgenic Poinsett76 cucumber cultivar was comparable to that of the CMV-resistant Market more. In actuality, Market had a higher amount of viral accumulation than the transgenic Poinsett76. Market more and transgenic Poinsett76 plants had an average infection rate of 3%, but untransformed Poinsett76 plants had an infection rate of roughly 72%. Compared to untransformed Poinsett76, fruit output was considerably higher in transgenic Poinsett76 and Market.

Maize: A member of the Potyvirus family, the Maize Dwarf Mosaic Virus (MDMV) is a significant cause of virus disease in maize and sorghum and is found all over the world. The virus, which can be found in nature in a number of different strains, impacts the growth and yield of both sweet and field corn varieties as well as the majority of sorghum genotypes. Murry et al. (1993) used particle bombardment or electroporation of sweet corn cell suspension cultures to create viable, transgenic corn plants that expressed the CP of MDMV. Transgenic plants showed a delay in the onset of symptoms and a decrease in symptom severity after being infected with MDMV when compared to untransformed controls.

In plants expressing the MDMV CP, challenge inoculation with the heterologous virus maize chlorotic mottle virus (MCMV) also induced a resistance response. When MDMV and MCMV were co-inoculated, plants that were transgenic for MDMV CP were able to confer resistance. Western blot analysis revealed that the MDMV CP also hampered MCMV replication. This strategy has the potential to support current approaches to treating maize viral infections.

Papaya: The papaya ringspot virus (PRSV) substantially reduces papaya yield. When papaya seedlings in Hawai, were inoculated with a mild isolate of the PRSV before they were

transplanted to the field, it was demonstrated that the use of traditional cross protection was successful. Additionally, Ling *et al.* (1991) demonstrated that transgenic lines of tobacco could withstand challenge PRSV inoculation. They did this by expressing the PRSV CP gene in tobacco. Fitch *et al.* (1992) used biolistic transformation of immature zygotic embryos to create transgenic papaya plants expressing the PRSV CP gene. One line of regenerated plants expressing the CP was entirely immune to virus infection, while other lines showed varied degrees of resistance.

Potato: Three significant potato virus diseases—potato leafroll virus (PLRV), potato virus X (PVX), and potato virus Y (PVY)- cause yield reductions of up to 50%. Kawchuk *et al.* (1990), Van Der Wilk *et al.* (1991), and Barker *et al.* (1992) introduced the CP gene of PLRV into the commercial cultivar. Desiree and Pentland Squire (1992) in comparison to non-transgenic controls, transgenic potato lines showed some resistance to PLRV infection and a delayed onset of illness. Although transgenic plants contained the CP's mRNA, no actual CP was found. Kawchuk *et al.* (1991) showed that the accumulation of CP mRNA in transgenic Russet Burbank was able to confer protection against PLRV, and the aphid transmission of the virus from transgenic plants was reduced com- pared with that in the controls. It appears that the mRNA alone was adequate to offer protection because the CP was not found in any of the transgenic strains. By specifically attaching to complementary viral RNA, the transgenic seems to prevent viral replication. The pattern and degree of resistance conferred by the transgene against inoculation by viruliferous aphids and in natural conditions showed great promise for the commercial application of this strategy to manage virus diseases of potatoes. Field testing and multi-location trials of transgenic potatoes expressing CP genes of PLRV, PVX, or PVY have been carried out independently by several groups. The cultivar Russet Burbank, which expresses the CP of potato virus S, was demonstrated to be resistant to challenge-inoculation by both the virus and viral particles by MacKenzie *et al.* (1991).

To create a potato cultivar with broad-spectrum resistance to viral infections, it might be able to combine the CP from the aforementioned viruses in a single plant (Lawson *et al.* 1990). By converting potato clones that were already naturally resistant to PLRV. Barker *et al.* (1994) introduced the CP gene of PLRV in an effort to combine host resistance with CPMP and further reduce the losses to PLRV in potato. PLRV barely accumulated to 1% of the amount reported in cultivars that were sensitive in transgenic plants with high levels of CP mRNA transcript levels, which were extremely resistant to the virus. Compared to either gene alone, the combination of the transgene and the host-derived resistance gene appeared to have an additive impact and imparted a higher level of resistance.

Rice: Significant yield losses in rice are brought on by several viral infections. For instance, rice stripe virus (RSV) is a significant barrier to rice production in many southern Asian nations.

RSV, a member of the Tenuivirus family, is spread by the brown plant hopper and has a segmented dsRNA genome. Rice protoplasts were electroporated with a cDNA clone containing the CP of RSV to undergo the transformation. Two Japanese rice types produced fertile plants once more. By inoculating plants with viruliferous plant hoppers, it was possible to test for RSV resistance in those that had been successfully trans- formed with the CP gene.

Tobacco: Although tobacco is widely debated and strongly opposed by the general public, it is nonetheless farmed as a significant revenue crop in both industrialised and developing nations. Due to its simplicity in transformation and re-generation, tobacco was used as a system in the original CAMP studies. These factors, along with the fact that tobacco serves as a host for numerous viruses, have led to tests of CPMP's efficacy using a variety of viruses on tobacco. It has been demonstrated in every instance that expressing a virus's CP gave either complete immunity or resistance (expressed as a delay in the onset of illness symptoms).

Tomato: Members of the tobamovirus family, including tomato mosaic virus (ToMV), cause substantial losses in tomatoes cultivated in greenhouses and outdoors. The virus is primarily transferred mechanically, such as infected instruments or the workers themselves in the greenhouse. Nelson *et al.* published the first field experiments of transgenic tomatoes expressing the CP of ToMV (1988). Further expressing TMV l-1 or ToMV in tomatoes, Sanders *et al.* (1992) tested the transgenic tomatoes for virus resistance in two separate places (Illinois and Florida, USA). Plants were intentionally inoculated with ToMV or TMV to test for viral resistance. Despite some lines having resistance to both viruses, resistance was more effective when homologous viruses were introduced to transgenic plants.

In addition to environmental factors, there were other factors that contributed to the plants' vulnerability to virus infection and subsequent yield reduction. The tomato yellow leafcurl geminivirus (TYLCV) is a significant hindrance to tomato cultivation in many tropical and sub-tropical regions of the world. The results showed that transgenic tomato plants expressing the ToMV CP hold great promise in the management of ToMV in greenhouse-grown tomatoes in the future. In tomatoes, Kunik *et al.* (1994) expressed the CP gene of the bi-partite DNA virus TYLCV. To test for symptom development and viral accumulation, virulifeous whiteflies were used to inoculate transgenic tomatoes. Some of the transgenic lines were resistant, expressed as a delay in disease development compared with that in untransformed controls. Whereas CP was detected in resistant transgenic lines, only the CP mRNA, not the CP, could be detected in transgenic lines that were as susceptible as the untransformed controls. This indicated that the production of CP is essential for the expression of resistance to TYLCV.

Numerous fruit, vegetable, and ornamental crops are infected by the tomato spotted wilt virus (TSWV), which is exceedingly challenging to control. The virus is distinct from other plant viruses in that it has a segmented, negative-sense RNA genome that is firmly packed with

nucleoprotein (N) subunits and enclosed by a lipid envelope. Thrips are the carrier of the virus. Tobacco was the first trans-gene plant to exhibit N-gene-mediated resistance to TSWV. Tomato and tobacco plants were given the N gene of a Hawaiian isolate of TSWV. Only 10% of the transgenic tomato plants displayed symptoms two weeks after infection (although all controls displayed symptoms), and only 30% to 50% of the several transgenic lines of tobacco displayed lesions when challenged with TSWV, compared to the controls.

In addition to the instances mentioned above, numerous researchers are working to create transgenic plants that express CP sequences obtained from viruses of many other crop plants, including citrus, grapes, and squash. One of the world's most important fruit crops, citrus fruit makes a considerable economic contribution to many national and regional economies (Behr *et al.* 1989). Citrus tristeza virus (CTV), which causes the most severe citrus virus illness, has already caused significant harm to the nations of Argentina, Brazil, Spain, and Venezuela's citrus sectors (Roistacher and Moreno 1991), and it poses a serious threat to the USA's citrus economy (Lee *et al.* 1992).

Various stages of testing for CTV resistance are being done on regenerated plants that express the CTV CP gene. Utilizing these three techniques of plant transformation, additional non-structural CTV genes are also being inserted into a variety of citrus genotypes. It will be interesting to find out if citrus, a woody perennial crop, can be genetically engineered to resist CTV and if the resistance will hold up under the variety of growing conditions and when citrus is repeatedly inoculated with different strains of CTV using highly effective natural aphid vectors.

Movment -Protein-Mediated Protection (CPMP)

Plant viruses can only travel from cell to cell when movement proteins (MP) are present. It has been demonstrated that these proteins alter how plasmodesmata perform their gating role, allowing virus particles or their nucleoprotein derivatives to move to neighbouring cells. In the beginning, this process was employed to create modified MP that are partially active as a transgene in tobacco, thereby engineering resistance to TMV. The conflict between the produced dysfunctional MP and the wild-type viruse ncoded MP for binding to the plasmodesmatal sites is thought to be the basis for the conferred resistance24, 25. Additionally, it was discovered that the aforementioned resistance worked against unrelated or distantly related viruses. For instance, the MP produced from the brome mosaic virus could be used to create resistance against TMV in tobacco, indicating functional conservation of the protein among several viruses26. In contrast to the single MP gene in tobamoviruses, viral movement is mediated by a set of three overlapping genes, known as the triple-gene-block (TGB) in potex-, carla- and hordei viruses. Expression of the modified central 12 kDa TGB gene of PVX, was shown to confer MP-derived resistance in potato to potexvirus PVX and carlaviruses potato virus M and potato virus S27. However, resistance was overcome when inoculated with viruse lacking a TGB, like PVY. This indicated

that the resistance depended upon the interaction of the viral derived and the transgene-derived MPs.

2. Replicase -Mediated Protection (RPMP)

To replicate and produce offspring viral RNA, plant viruses encode an RNA-dependent RNA polymerase. This stage in the viral replication process is crucial. When challenged by the homologous virus TMV Ul, the transgenic tobacco harbouring the replicase gene construct displayed a very high level of resistance or complete immunity. The resistance was superior to CPMP in that it was effective against viral and viral RNA infection, could sustain extremely large inoculum levels, and worked even at relatively high temperatures. Studies using 54-kDa gene-expressed protoplasts from plants have demonstrated the importance of protein expression in inducing the host's protective state against viral infection.

Carr and Zaitlin explain the molecular underpinnings of the resistance response in great depth (1993). Following the report of replicase-mediated resistance against TMV infection, numerous groups with different virus-host combinations achieved comparable results. Using a cDNA clone that represented a shortened version of the CMV 2a protein, Anderson *et al.* (1992) created a plant transformation vector. This protein is thought to be involved in virus replication based on similarities in amino acid sequences. The shortened protein was expressed in transgenic tobacco plants that were extremely resistant to mechanical inoculation with CMV and CMV RNA. Two groups have looked into how resistance to PVX is affected by the replicase gene of pVX. Braun and Hemenway (1992) constructed clones representing either the full-length or the amino-terminal portions of the replicase gene of PVX and transformed tobacco with them.

The lines expressing different lengths of the replicase gene displayed varying degrees of resistance to PVX infection. There were fewer local lesions and less virus accumulation in the resistant lines, which expressed either the entire replicase gene or specific regions of it, compared to the untransformed controls. Replicase-mediated protection was discovered to be more efficient with PVX than CPMP. Longstaff *et al.* (1993) examined the function of the conserved GDD motif in inducing the resistance response by expressing parts of the PVX replicase gene that had previously been altered by inserting mutations into the motif. According to their findings, lines that expressed the replicase gene with various alterations displayed either full protection or none at all.

In the cases of TMV, CMV, PVX, CyRSV, and I'EBV, replicase-mediated protection appears to be quite effective, and it has the potential to provide effective control of other virus-host combinations involving commercially significant agricultural plants. However, the production of short RNA2 sequences encoding for the putative replicase proteins of AlMV and BMV in transgenic plants did not impart resistance to challenge-inoculation.

Resistance through expression of antisense RNA or untranslatable RNA of viral origin:

Several groups have looked into viral sequences with antisense orientation or with sense orientation but untranslatable RNA, for their ability to confer resistance to virus infection, whereas the majority of examples of genetically engineered protection against viruses used CP-coding sequences in the sense orientation. In the Russet Burbank potato, Kawchuk *et al.* (1991) discovered that the antisense RNA of PLRV CP afforded protection similar to that of the sense construct. No CP was found, even in plants transformed with the translatable sense construct, indicating that the resistance was likely caused by the transcript's presence alone and was independent of CP formation.

The RNA transcript appears to accumulate to a sufficient level to prevent the challenge virus from replicating at a particular stage, most likely by binding only to the incoming viral RNA. With regard to TSWV, plants transformed with a translationally hindered gene construct had resistance levels comparable to those of CP-expressing lines, showing that the resistance was brought on by antisense RNA-mediated inhibition of virus multiplication. When used against viruses that replicate in the nucleus, such as the DNA-containing tomato golden mosaic geminivirus, antisense-RNA-mediated defence may be more effective (TGMV). Day *et al.* (19%) made use of a gene construct that expressed the TGMV gene called ALI in the antisense orientation, which is necessary for virus replication. Transgenic tobacco plants exposed to a challenge-inoculum displayed reduced viral replication and milder symptoms. The above results show that use of antisense or impaired sense RNA technology has considerable potential in reducing the impact of some viruses, perhaps especially those confined to phloem tissues or those that replicate in the nucleus.

Expression of satellite RNA sequences:

Some plant viruses co-occur in strange interactions with small RNA molecules that depend on the helper virus's reproduction. The helper virus does not require these tiny RNAs, commonly known as satellite RNAs (SatRNA), to replicate, and their genomes do not share many similarities. SatRNAs differ from satellite viruses in that they do not encode coat protein, contain the helper virus' DNA, and co-transmit with the helper virus (Matthews 1991). According on the helper virus strain and the host plant, it has been shown that satRNAs can modify the disease caused by the helper virus by either making the symptoms more severe or less severe (Kaper and Collmer, 1988).

The ability of some satRNAs to lower illness severity by interfering with the reproduction of their helper viruses has been used in the field to treat viral infections. The application of symptom-attenuating satRNA in transgenic plants and the mechanism of attenuation are recent subjects that have been studied (Yie and Tien, 1993). By employing transgenic tobacco plants, Harrison *et al.* (1987) showed that expression of the CMV satRNA

resulted in lower levels of challenge (helper) virus accumulation than those in untransformed controls. Resistance, which was also exhibited as a reduction in symptoms, was seen to be effective against vaccination using the aphid vector.

Transgenic tomato plants that produced the CMV satRNA in the field showed a significant decrease in disease severity and a 50% increase in fruit yield when compared to untransformed controls. Tobacco and tomato plants cannot be completely protected from naturopathic illnesses, according to field tests done in China. Yie *et al.* (1992) tested the viability of combining two various methods of inducing protection in transgenic plants by expressing the CMV CP gene as well as the symptom-attenuating satRNA in transgenic tobacco. The field testing revealed that plants that produced both CP and satRNA had twice as much resistance to CMV infection than plants with only one of the two genes.

Resistance mediated by the expression of other viral and non- viral sequences:

Recent efforts to achieve virus resistance have involved alternative strategies, such as the expression of defective viral movement protein, viral protease, defective interfering RNA, virus specific antibody genes, antiviral pokeweed protein and interferon. The 30-kDa protein encoded by TMV has been shown to be involved in cell-to-cell movement of the virus, modifying the plasmodesmata. If this movement protein is rendered non-functional, the virus may not be able to move from the initially infected cells, thereby limiting virus spread. Malyshenko *et al.* (1993) expressed the 30-kDa protein of one mutant strain of TMV, the temperature sensitive Ni2.519, in transgenic tobacco and demonstrated reduced accumulation of wild-type temperature-resistant TMV. They also showed that the putative movement protein from an unrelated virus, BMV, conferred resistance to TMV when expressed in tobacco.

Transgenic Xanthi NN tobacco plants were resistant to challenge-inoculation by TMV and TMV RNA even though they expressed the 30-kDa protein but lacked amino acids 2 to 5. Although significant levels of the wild-type 30-kDa protein accumulated in the plasmodesmata, the modified 30-kDa protein was only found in significantly smaller quantities. In addition, the plants were resistant to the sun hemp mosaic virus and the tobacco mild green mosaic virus. Additionally, the plants resisted the systemic buildup of viruses from other virus groups, including the DNA-containing peanut chlorotic stripe caulimovirus, AlMV, and tobacco rattling virus. One function that is shared by all viruses examined was thought to have been hampered by TMV's defective movement protein. This offers the appealing prospect of employing just one viral gene.

Some plant viruses also encode proteases, which convert the polyproteins generated by the virus into a variety of useful gene products. The tobacco vein mottling virus (TVMV genome-linked) protein protease-coding areas were expressed in tobacco by Maiti *et al.* in 1993. Transgenic lines that were challenged with an infection did not exhibit any symptoms. The

resistance, however, was no longer effective against PVY and the tobacco etch virus, two other potyviruses. When challenged by the aforementioned three potyviruses, plants that expressed the TVMV CP or the gene encoding for cylindrical inclusion protein (CI) responded in distinct ways. CI-expressors were vulnerable to all three potyviruses, whereas CP-expressing plants were resistant to each one.

The defective, interfering RNAs (DI RNA) of a plant virus were described by Hillman *et al.* (1987). DI RNAs are truncated RNA molecules that represent portions of the viral genome and are often responsible for the amelioration of symptoms caused by the 'parent' virus. Kollar *et al.* (1993) cloned DI RNA of CyRSV and showed that the in-vitro synthesized RNA transcript was biologically active and, when co-inoculated with CyRSV, reduced the symptoms of CyRSV. Transgenic *N. benfkmniuna* plants expressing DI RNA displayed resistance to CyRSV infection and were protected from severe stunting and apical necrosis.

Protection was probably achieved due to the ability of DI RNA to interfere with replication of CyRSV. This form of virus-derived protection offers an additional strategy for the protection of plants from virus infection.

Another way to confer viral resistance in agricultural plants has recently been successful expression of biologically active anti-bodies in transgenic plants (plantibodies). Raising monoclonal antibodies against the artichoke mottled crinkle virus (AMCV), Tavladoraki *et al.* (1993) selected the monoclonal (Fs) with the best affinity for the coat protein. The heavy and light immunoglobulin chains' cDNA clones were made and put into expression in Escherichia coli. Variable domains of the light and heavy chains produced in E. coli have a weaker affinity for the virus than Fs. The ability of the cloned antibody gene to "neutralise" the virus in transgenic tobacco plants was then assessed by Tavladoraki *et al.* in 1993.

Transgenic lines that had been injected with AMCV showed resistance to virus infection, delayed illness onset, and decreased virus titres as compared to untransformed controls. Transgenic plants were found to be equally vulnerable to infection as control plants after being inoculated with the heterologous virus CMV. This was the first instance of plantibodies being used to prevent viral reproduction. In order to create transgenic grapefruit that expresses antibody genes unique to CTV proteins, we are attempting a similar methodology. More "neutralising" antibody molecules must be produced due to the high viral CP molecule synthesis in infected cells. If the antibodies are targeted against the more uncommon viral gene products seen in infected host cells, such as replicase or protease, the plantibody method may be more successful. Two recent reports demonstrate that the source of resist- ance genes need not necessarily be limited to plant viruses. Lodge *et al.* (1993) utilized the antiviral properties of a ribosome-inactivating protein isolated from pokeweed. A cDNA clone coding for the pokeweed

antiviral protein (PAP) was introduced into tobacco and potato plants by *Agrobacterium*-mediated transformation.

The amount of PAP produced varied significantly amongst the various transgenic lines. When challenged with viruses from diverse groups such as PVX, PVY and PLRV, the PAP-expressing plants exhibited significant levels of resistance, manifested as delays in disease development and reduction in virus accumulation. The PAP-mediated protection seems to be effective against diverse groups of plant viruses and holds promise as another strategy to introduce virus resistance into crop plants. Truve *et al.* (1993) 'expressed a cDNA clone of murine 2-5A synthetase gene in potato. The gene is responsible for creating an antiviral state, mediated by interferon, in mammalian cells. Transgenic potato plants expressing the 2-5A synthetase were less susceptible to infection with PVX than untransformed controls. It was suggested that the expression of interferon may confer a generalized, broad-spectrum resistance to diverse plant viruses in crop plants and postulated that the expression of 2-5A synthetase might have elicited an antiviral environment analogous to that existent in mammalian cells (Truve *et al.* 1993). Further studies should reveal the effectiveness and the molecular basis of such interferon-mediated resistance to plant virus infection.

Powell-Abel *et al.* initially revealed CPMP) since the hunt for genetically engineered virus resistance in plants (1986). Based on a solid foundation of knowledge and quick advancement in fields like plant virology, plant physiology, biochemistry, and molecular biology, this has encompassed the use of viral as well as non-viral genes. Transgenic lines of several agronomically significant crops resistant to several plant viruses have either already field tested or are in various stages of field evaluation, particularly in the case of CPMP.

Our growing understanding of the fundamental mechanisms underlying virus resistance, the molecular interactions between the challenge virus and the transgene, and the plant factors involved in virus replication will undoubtedly result in the development of even more exciting and successful avenues for the management of plant virus diseases. Future research will determine the true impact these modern approaches can have on disease prevention, particularly in terms of enhancing and stabilising the agricultural productivity of emerging nations (Moffat 1994). While the majority of biotechnology methods used to improve crops in affluent nations aim to lower production costs, the protection of crops from diseases (and hence maximising yield) is a top goal for agricultural organisations in developing nations. It won't be long until plants are genetically modified.

Table 1. Summary of plant species and viruses for which genetically engineered resistance has been demonstrated.

Resistance mediated by:	Plant	Virus(es) resisted	Reference(s)
Coat protein	Alfalfa	AlMV	Hill *et al.* (1991), Van Dun *et al.* (1987)
	Barley	BYDV	Lister *et al.* (1994b)
	Cucumber	CMV	Gonsalves *et al.* (1992)
	Maize	MDMV	Murry *et al.* (1993)
	Oats	BYDV	Lister *et al.* (1994a)
	Papaya	PRSV	Fitch *et al.* (1992)
	Potato	PLRV	Kaniewski *et al.* (1994)
		PVX	Hoekema *et al.* (1989), Lawson *et al.* (1990), Jongedijk *et al.* (1993), Kaniewski *et al.* (1993)
		PVY	Kaniewski *et al.* (1989), Lawson *et al.* (1990)
		PVS	MacKenzie *et al.* (1991)
		PVM	Mackenzie *et al.* (1991)
	Rice	RSV	Hayakawa *et al.* (1992)
	Tobacco	TMV	Powell-Abel *et al.* (1986)
		TSWV	Kim *et al.* (1994)
		PRSV	Ling *et al.* (1991)
		CMV	Cuozzo *et al.* (1988)
		AlMV	Loesch-Fries *et al.* (1987), Tumer *et al.* (1987), Anderson *et al.* (1989), Van Dun *et al.* (1987)
	Tomato	TMV	Nelson *et al.* (1988), Sanders *et al.* (1992)
		AlMV	Tumer *et al.* (1987)
		ToMV	Sanders *et al.* (1992)
		TSWV	Gielen *et al.* (1991), De Haan *et al.* (1992), MacKenzie & Ellis (1992), Kim *et al.* (1994)
		TYLCV	Kunik *et al.* (1994)
Antisense/untranslatable RNA	Potato	PLRV	Kawchuk *et al.* (1991)
	Tobacco	Potyviruses,TSWV	De Haan *et al.* (1992)
	Tobacco	TGMV	Day *et al.* (1991)
Satellite RNA	Tobacco	CMV	Harrison *et al.* (1987)
	Tomato	CMV	Yie & Tien (1993)
Coat protein and satellite RNA	Tobacco	CMV	Yie *et al.* (1992)
Replicase-associated sequences	Tobacco	TMV	Golemboski *et al.* (1990)
		CMV	Anderson *et al.* (1992)
		PEBV	MacFarlane & Davies (1992)
		PVX	Braun & Hemenway (1992)
		CyRSV	Rubino *et al.* (1993)
	Potato	PVY	Chiang *et al.* (1994)
Viral movement proteins	Tobacco	TMV	Malyshenko *et al.* (1993) Lapidot *et al.* (1993) Cooper *et al.* (1994)
Viral proteases	Tobacco	TVMV	Maiti *et al.* (1993)
	Potato	PVY	Chiang *et al.* (1994)
Defective, interfering RNA	Tobacco	CyRSV	Kollar *et al.* (1993)
Plantibodies	Tobacco	AMCV	Tavladoraki *et al.* (1993)
Pokeweed proteins	Tobacco	PVX	Lodge *et al.* (1993)
	Potato?	PVY	Lodge *et al.* (1993)
		PLRV	Lodge *et al.* (1993)
Animal interferon	Potato	PVX	Truve *et al.* (1993)

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Chapter 8 EXPLOITATION OF CHITINASE GENES IN PLANT DISEASE RESISTANCE

Shaik Munnysha, Manisha, Ramavath Abhi and Nitisha Gahlot

Introduction:

After cellulose, chitin is the polysaccharide that occurs in nature in the greatest abundance. Chitin is a 1,4-N-acetylglucosamine linear polymer (GlcNAC). It is a polysaccharide that is white, rigid, and inelastic. The acetoamido group replaces the hydroxyl group in the cellulose-like structure of chitin. An enzyme called chitinase can break down chitin. The breakdown of chitin occurs in two steps: first, chitinases cleave the polymer of chitin into chitin oligosaccharide, and then chitobiases further cleave it into N-acetylglucosamine and monosaccharide.

History:

1811 Chitin was first discovered by Professor Henri Braconnot, who isolated it from mushroom and name it "Fungine". Hofmann determined the structure of chitin in 1929. 1859 - Rought discovered chitosan, a derivative of chitin. Chitinases were first discovered by Bernald on orchid bulb as an antifungal factor (Sharma *et al.*, 2011). 1977- First international conference on chitin\chitosan.

Structure of chitin

Exists in three different forms: α-chitin, β-chitin and γ-chitin. α-chitin is the most abundant, isomorphic and more compact form due to arrangements of chitin chain in antiparallel fashion. β-chitin is loosely packed as a chitin chains in parallel fashion. γ-chitin is a mixture of both α-chitin and β-chitin

Chitinases

Chitinases are enzymes (glycosyl hydrolases) present in wide range of organisms. Chitinases have the ability to degrade chitin directly to low molecular weight chitooligomers. Chitinases have been divided into 2 main groups- Endochitinases andExo-chitinases. Endochitinases cleave chitin at internal sites of N-acetyl-D-glucosamine (GlcNAc or NAG). Exochitinase catalyze chitin progressively to produce NAG, chitobiose or chitotriose.

Major groups of chitinases:

Arthropods, yeast, bacteria, fungus, and plants. Bombyx mori and Manduca sexta are two insect chitinases. Rice, bean, barley, cabbage, carrot, cucumber, garlic, oats, onion, tomato, pea, and peanut all contain plant chitinases. *Bacillus pumilus, Aeromonas punctata, Xanthomonas maltophila, Paenibacillus illinoisensis, Streptomyces thermoviolaceus,* and *Streptomyces*

violaceusniger are among the bacteria that produce chitinases. *Trichoderma* sp., *Taloromyces flavus, Candida albicans, and Saccharomyces cerevisiae* all produce fungus-specific chitinases.

Shape and size of chitinases

3-D structures. Chitinases are glycosyl hydrolases with the sizes ranging from 20 kDa to about 90 kDa, Insect chitinases: 40-85 kDa, Fungi: 27-190 kD, Plant: 25-40 kDa and Bacteria: 20-60 kDa.

Classification of plant chitinases

Plant chitinases are often categorised into five classes based on how similar their amino acid sequences are; Class 1 chitinases have the CBD at the N-terminal region. The chitin binding domain is absent from classes II and III. Two CBDs and a C-terminal extension are present in Class V. In Class IV chitinases, there was only one half of the CBD present. Plant chitinases have a single catalytic domain that makes up the majority of their molecular structure.

Yeast chitinases contain four domains

Signal sequence, catalytic domain, serine\threonine rich region, C-terminal chitin-binding domain.

Fungal chitinases consists of five different domains

N-terminal signal peptide region, catalytic domain, chitin binding domain, serine\threonine rich-region and C-terminal extension region.

Groups of chitinases

Based on amino acid similarity, chitinases categorized into 2 families:

Family 18 -This family consists of class III and class V chitinases. Found in wide range of organisms including bacteria, plants animals and fungi.

Family 19: This family consists of class I, II and IV chitinases. These are found only in plants and *Streptomyces* sp.

PR proteins: Pathogenesis-Related Proteins**:** Stress proteins**.** There are 17 PR families**.** PR proteins associated with the development of Systemic Acquired Resistance (SAR) against further infection by fungi, bacteria and viruses. Found in many plant species belonging to various families

Genetic engineering for plant disease resistance

Genetic engineering: is the artificial manipulation or alternation of genes. Genetic engineering involves: Removing a gene (target gene) from one organism, inserting target gene into DNA of another organism. This method is called ‘cut and paste ‘process.

Development of transgenic plants

Transgenic plants are those whose genomes have been modified by the insertion of one or more transgenes. A target genome, a vector to carry the gene, modification of foreign DNA to increase the level of gene expression, a method to deliver plasmid DNA into the cell, a method to

identify the transformed cell, and tissue culture to recover viable plants from the transformed cells are the basic requirements for transformation.

Characteristics of vector: (*Agrobacterium tumefaciens*)

Natural genetic engineer, Ti plasmid (200 kbp), T-DNA (20 kb), Genes for hormone and opine biosynthesis, Vir region (30 kb), 24 vir genes code for proteins that function in transfer of T-DNA into plant cells

Development of disease resistant plant with chitinase transgene

1. Chitinase gene isolated from a source
2. Put in a T-DNA region of Ti plasmid
3. Tobacco plant cells transferred
4. Tissue culture protocol
5. Transgenic tobacco plant with resistant to the attack of *Rhizoctonia solani*

Biocontrol strategies:

Engineered plants with overexpression of chitinases

The current model of plant pathogenesis concentrates on creating disease-resistant transgenic plants by including genes that code for chitinase. Tobacco plants were given the bean chitinase gene to increase their resistance to *Rhizoctonia solani*. Sheath blight resistance is provided by transgenic rice plants bearing the rice chitinase gene and the TLP gene. Chitinase genes from biocontrol fungi like Trichoderma are unquestionably superior to those from the comparable plant pathogens in terms of antifungal activity. Plant chitinases typically only influence the hyphal tip and cannot break down more difficult chitin complexes. Crop plants should have a high level of resistance to a range of fungal diseases resistance to these chitinase genes.

Exploitation of chitinase gene (*Trichoderma* sp.) in tobacco plants:

Rhizoctonia solani and *Alternaria alternata* are two major pathogens of tobacco. *Rhizoctonia solani:* Target leaf spot and damping off. *Alternaria alternata:* Alternaria leaf spot\ brown spot.

Chitinase gene expression and application

Gene	Origin	Application
Maize chitinase 2 gene	Zea mays	Effective against rot pathogen (*Fusarium* sp.)
Rice chitinase (chi 11) gene	Rice	Reduce sheath blight
Class 2 endochitinase gene	Barley	Inhibit growth of A. solani
Rice class 1 chitinase gene	Rice	Resistant against leaf spot
Rice chitinase 3 gene	Rice	Resistant against leaf spot in peanut

Expression of chitinase in transgenic plants

Gene	Source	Transgenic crops	Role
Chitinase RCH 10	Rice	Lilium oriental	Resistant against Botritis cinerea
Chitinase chit 33	*Trichoderma atroviride*	Brassica napus	Resistant against stem rot disease
Endochitinase gene	*Trichoderma harzianum*	Guava	Resistant against wilt
Chitinase gene (CaMV-Ubi-chi 1)	Rice plant	Grapevine	Resistant against powdery mildew
Chitinase RCC11	Rice	Litchi	Resistsnt against die-back, leaf spots and blights

Applications

Waste management: Preparation of single cell protein and treatment of chitinous waste from sea food industry.

Biocontrol agent: Biological control of phytopathogenic fungi and it is used as a biopesticide against various insects and pests.

Medical appilication: Chitooligomers can be used as antitumor agents, dietary fibres and antihypertensive agents and chitinase can be used as antifungal agents and it also plays a crusial role in asthma.

Future prospectus:

Chitinases have many uses, including the creation of transgenic plants and the biocontrol of plant diseases. A deeper understanding of chitinase genes and their biotechnological uses in agriculture and other industries will come from more research on microbial chitinase employing genetic engineering methods. The use of microbial chitinases in the industrial and biotechnological fields offers considerable promise.

Conclusion:

It has been established that antifungal genes, such as chitinase, are promising candidate genes for the efficient management of fungal infections in transgenic crop plants. Future crop plants should be developed using these genes to become more fungus resistant. By shielding crop plants from fungus diseases, this will significantly boost agricultural production.

Chapter 9 SEROLOGICAL TECHNIQUES FOR DETECTION OF VIRUS

Shaik Munnysha, Manisha, Nitisha Gahlot and Ramavath Abhi

Introduction of serology:

It is a method for detecting and identifying antigenic substances and the organisms that carry them by combining the actions of the antigen (virus) and its antibody (antiserum).

To effectively control plant viruses, it is crucial to identify them as soon as possible. The diagnosis of viral infections is becoming more crucial since free trade agreements (FTAs) have accelerated global trade and the rapid climatic change encourage the transmission of viruses, their hosts, and their vectors from one country to another. A clinical diagnosis may not be suitable since the symptoms of viral infections are not always distinct and can be mistaken with those of abiotic stressors. Enzyme-linked immunosorbent assays (ELISAs), which were created using the serological principle, have been in use for the past three decades. However, due to various restrictions such the lack of an antibody for the target virus, the high cost of producing an antibody, the need for a large volume of samples, and the length of time required to perform an ELISA, the effectiveness of ELISAs to detect plant viruses is decreasing. There are many cutting-edge methods for getting around ELISA's shortcomings. The polymerase chain reaction (PCR), which was initially created as a method to amplify target DNA, has since expanded into a wide variety of variants with greater sensitivity than ELISAs. This article reviews a variety of plant virus detection systems, including immunological-based detection

Serological techniques

Types of Antigen –antibody reactions

Conventional techniques:

- Precipitation reaction
- Agglutination reaction
- Complement fixation test
- Neutralisation test

Newer techniques (Labelled assays):

- Precipitin tests
  - Tube precipitation test
  - Tube (ring) precipitation
  - Micro precipitin Test
- Precipitin Test in Agar (Gel diffusion Test)
- Chloroplast agglutination test
- Latex agglutination test

- Enzyme linked immunosorbent assay (ELISA) DAS ELISA, Indirect ELISA, Competitive ELIZA
- Immunofluorescnce assay (IFA)
- Radioimmunoassay (RIA)
- Chemiluminescence – linked immunoassay (CLIA)
- Rapid test – Cassette ELISA, Lateral flow test (Immunochromatographic test), Flow through assay

Definition:

When a soluble antigen combines with a particular antibody in the presence of an electrolyte at the right temperature and pH, an antigen-antibody complex forms in the form of insoluble precipitate bands or precipitate rings depending on whether a liquid media is utilised or a gel-containing medium.

Precipitation Test:

A) Antigen and antibody combine to generate a precipitate when combined. In plant virology, this precipitin is frequently employed. The amount of precipitate that forms depends on a variety of variables, including the concentration of salt, the pH, the temperature, the presence of interfering chemicals, and the ratio of antibody to antigen concentration, which is crucial because an excess of either can inhibit precipitation.

B) Precipitation in Liquid medium

- Ring test – Ascoli's thermoprecipitin test, Streptococcal grouping by Lancefield's technique
- Slide test - Flocculation test – VDRL test for syphilis
- Tube test - Kahn's test for syphilis

C) Precipitation in gel (Immunodiffusion)

- Single diffusion in one dimension (Oudin Procedure)
- Double diffusion in one dimension (Oakley Fultrope procedure)
- Single diffusion in two dimensions (Radial immunodiffusion)
- Double diffusion in two dimensions (Ouchterlony procedure)

D) Immunoelectrophoresis

Principle of ELISA

ELISA can provide a useful measurement of antigen or antibody concentration.

There are two components

Immunosorbent: An absorbing material (polyvinyl, polystyrene) is used that specifically absorbs the known antigen or antibody present in serum (delete)

Enzyme: Is used to label one of the components of immunoassay.

Following antigen- antibody reaction, chromogenic substrate specific to enzyme alkaline phosphatase) is added. Reaction is detected by reading optical density.

(Ag+AB complex) - Enzyme + substrate ----> activates the chromogen--->colour change ----> Detected by spectrophotometry

Types of ELISA:

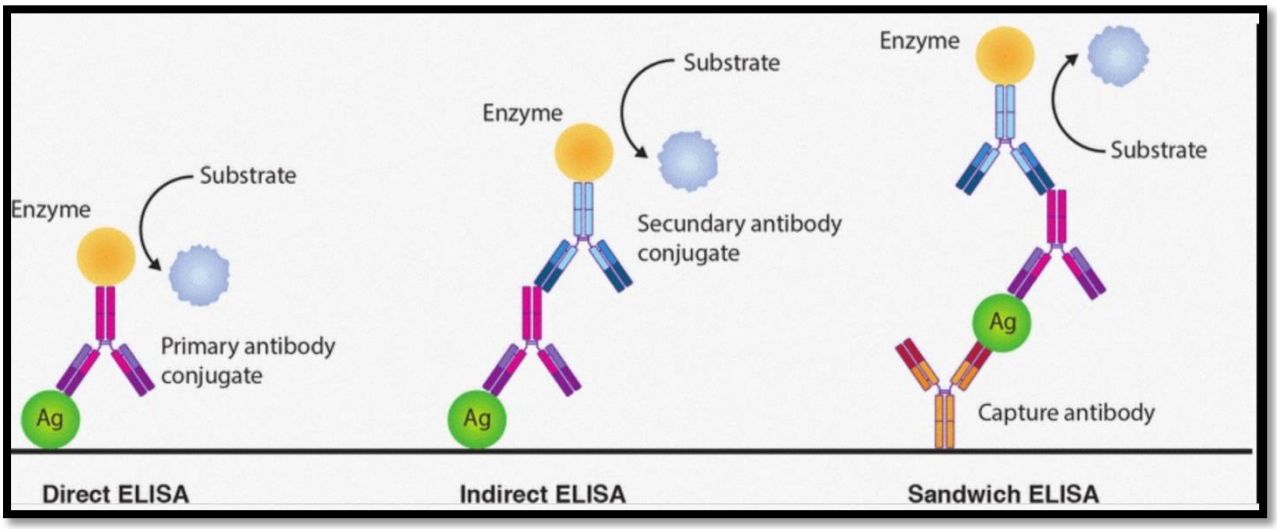
Direct ELISA - used for detection of antigen

Indirect ELISA- used for detection of antibody/ antigen

Sandwich ELISA - used for detection of antigen -Direct (single Ab) and Indirect (double Ab) Sandwich ELISA

Competitive ELSA - used for detection of antigen/ antibody

Cassette ELISA (Cylinder ELISA)- used for detection of antibody Applications For Antigen detection : Hepatitis B srface antigen, hepatitis B inner core antigen, NS1 dengue antigen For Antibody detection : Hepatitis B, C, HIV, Dengue, Epstein Barr virus , Herpes Simplex virus , Toxoplasmosis, Leishmaniasis



Serological methods:

Systems for serological detection rely on particular antibodies created by animals in response to antigens. If viral antigens are utilised to create antibodies, viruses can be found. These methods have really been employed as a standard diagnostic tool. Numerous serological techniques, such as the enzyme-linked immunosorbent assay (ELISA), the tissue blot immunoassay, and quartz crystal microbalance immunosensors, have been reported (QCMI)

ELISA

Common ELISAs are carried out in polystyrene plates that can bind proteins or antibodies in conjunction with the enzyme-substrate reaction. The timing and development

conditions of the enzyme-substrate reaction must be tuned in order to obtain a precise and repeatable result. The widely used ELISA technique has been used to identify plant viruses in plant material, insect vectors, and seeds. Level of infection is measured based on the optical density (the degree of coloration) of ELISA reaction. Advantages of ELISA are that it is sensitive, a great number of samples can be examined at the same time. little amount of antibody for the detection of diseases, and the process can be semiautomate. Specific antiserum has been developed against the target virus. It has been employed for the detection of a lot of viruses including CMV, Citrus tristeza virus (CTV), Potato leaf roll virus (PLRV), Potato virus X (PVX), and Potato virus Y (PVY). Large amount of sample for ELISA is needed for capturing antigen of interest from the sample compared to sample requiring for molecular methods and it takes about 2 days for diagnosis. Since ELISA is antibody-antigen based assay, availability of antibody properly responding against the target agent is regarded as very important factor. ELISA often offers misdiagnosis due to false positive which is mainly resulted from non-specific reactions or cross-reactivity with certain factors in samples. Antibody used in ELISA can respond to many strains with an obvious different symptom because of lack of specificity. Therefore, strains of virus very related cannot be differentiated correctly by ELISA. Although ELISA sensitivity was increased by adding some additives in extraction buffer, ELISA is generally less sensitive when compared to molecular methods. Because of these reasons, although ELISAs have been widely used for diagnostic purpose up to date, the use of ELISA in terms of diagnosis seems to be gradually decreased. It is thought that alternative tools to be employed in coming age will be introduced in to a diagnostic market or more researches will be continued to overcome ELISA's shortcomings.

Tissue blot immunoassay (TBIA)

TBIA has the same reliability as ELISA to identify plant viruses since its operating principle is the same as that of ELISA, to which an antibody is applied. The main distinction is that while TBIA is carried out on nitrocellulose and nylon membranes, ELISAs are performed on polystyrene plates. This is the reason why the test is known as TBIA or TIBA. Similar to ELISA, TBIA also requires a particular antibody to prevent false positive results and a high viral concentration to prevent false negative results. TBIA, however, offers ELISA significant advantages in terms of detection time, cost, sensitivity, and simplicity. it has been applied for diagnosis of a number of viral diseases caused by Bamboo mosaic virus (BoMV), Bean yellow mosaic virus (BYMV), CTV, Cymbidium mosaic virus (CyMV), Papaya ringspot virus (PRSV), Sweet potato feathery mottle virus (SPFMV), and Tomato spotted wilt virus (TSWV).

Quartz crystal microbalance immunosensors (QCMI)

The QCM has been extensively used to measure tiny masses in vacuum, gas, and liquid conditions in real time based on vibrations and frequency changes. QCMI functions as a mass-

sensitive transducer when QCM and immunological techniques are combined. In a positive reaction, antigen-antibody binding reaction reduces quartz crystal oscillation frequency. The features of QCMI, such as its high sensitivity, real-time output, portability, label-free entities, and cheap operational, manufacturing, and maintenance costs, make it an appealing alternative to traditional analysis techniques. By adding the signal enhancement step, the detection sensitivity can be raised if the analytical signal is too weak to detect the target components. It is possible to identify plant viruses on-site using a detection tool for QCM that is sterile and QCM coated with virus-specific antibodies to detect plant viruses. Since QCM has shown to be effective at detecting plant viruses, others such as Cymbidium mosaic virus (CyMV), Turnip yellow mosaic virus (TYMV), and TMV were also discovered using QCM.

Molecular methods:

Molecular methods can be applied for diagnosis of many viral diseases when genetic information of viruses is available. As an alternative method to serological one, it is most commonly used in the laboratory due to high accuracy and sensitivity.

Polymerase chain reaction (PCR).

Reverse transcription and PCR PCR (RT-PCR). A specific DNA sequence can be amplified, or produced in millions of identical copies, using the PCR process inside a tiny reaction tube. Each time a new round of DNA amplification is started, the complementary strand of DNA is denatured before two sets of oligonucleotides, or primers, anneal to it. The DNA polymerase is then guided by primers to create new DNA. Every reaction happens in a template-dependent order. Numerous molecular biology-based research applications, including cloning, gene modification, gene expression analysis, genotyping, sequencing, and mutagenesis, have made use of PCR as one of their primary tools. PCR is currently a well-liked method for detecting plant viruses in the lab and is widely applied in molecular investigations.

Multiplex PCR.

PCR in multiplex. Multiplex PCR allows the simultaneous detection of two or more targets' DNA or RNA in a single reaction. For this approach to detect more than two viruses or bacteria, it needed numerous specialised primers. There are numerous instances of viruses and other plant diseases being found in one host at the same time. Through multiplex-PCR, the numerous main viruses were simultaneously found infected apple trees. Apple chlorotic leafspot virus (ACLSV), Apple mosaic virus (ApMV), Prune dwarf virus (PDV), Prunus necrotic ringspot virus (PNRSV), and Plum pox virus (PPV) infection rates were compared between multiplex-PCR and ELISA to detect plant viruses. While the same samples were used, multiplex-PCR showed a higher infection rate of about 16.7% while ELISA only detected 10%, proving that it was superior to ELISA in time of detection and in sensitivity.

Nested PCR

When the viral titre is extremely low, the target gene is unstable, and electrophoresis is not an option due to the poor amplification product, the approach is helpful. For a second PCR amplification, the primary PCR amplification product is employed. The risk of contamination may, however, be a factor in the second reaction. Nested PCR can be used to tackle the issues listed above. By using this method, several viruses, including PNRSV, PDV, PPV, and CTV, were discovered. The combination of this nested PCR and Immunocapture-RT PCR increased sensitivity and facilitated sample preparation. The Lettuce Mosaic Virus (LMV) was found using this technique, even in individual aphids.

Co-operational PCR (Co-PCR)

A tetra primer set is necessary for both nested-PCR and cooperative PCR. But compared to nested-PCR, which requires two external and two internal primers, cooperative PCR only needs one exterior and three internal primers. Cooperative PCR has several advantages over traditional PCR because it uses four primers, just like nested-PCR. Benefits include a single reaction, reduced chance of contamination, high sensitivity akin to nested PCR, real-time detection, and dot blot hybridization coupling capabilities. Additionally, cooperative PCR can prevent false positives from being detected by nested PCR and can be applied to capillary air thermal cyclers, which cannot be used with nest-PCR to detect the squash vein yellowing virus (SqVYV). The existence of PCR inhibitors is the main barrier to the use of traditional PCR. This issue can be overcome by co-PCR with diluted samples.

Real-time PCR. (RT PCR)

This was created as one of the technical approaches to accurately quantify PCR results as well as monitor the amplification products of PCR in real-time. Real-time PCR has a significantly shorter detection time and can be utilised for target genes in low concentrations, making diagnosis possible without the requirement for gel electrophoresis. Additionally, it is well recognised that there is a lower chance of contamination and it is faster than traditional PCR. Although the real-time monitoring curve increased as the DNA amplified exponentially, real-time PCR has significant limitations. One is that when it reaches a specific level or plateau, the amplification stops. Real-time methods have the additional drawback of requiring very expensive equipment. Despite these drawbacks, real-time PCR has been increasingly used because this method has been showed valuable detection for plant viruses. Citrus tristeza virus (CTV) in different plant tissues and TMV in soil were detected by real-time PCR and quantified Citrus leaf blotch virus (CLBV). It also used to discriminate two potato pathogenic bacteria on infected potato tubers.

Loop-mediated isothermal amplification (LAMP)

The four primers are used in LAMP, which is carried out for an hour at a steady temperature. DNA has continuously amplified from the first products, resulting in a variety of sized DNA structures. The first product is created in the loop formation. Therefore, even though the target gene is present in extremely little quantities, the diagnosis is still attainable. By using electrophoresis, the LAMP reaction products can be found in a smear of different bands in a positive LAMP reaction lane. The LAMP test has recently been used for the quick detection of a number of animal viruses, including canine parvovirus. It has also been applied to the identification of Phytoplasmas, GMOs, and the sex of asparagus. The RT-LAMP has been created for straightforward RNA virus monitoring, including PVY and PLRV.

Microarray (Oligonucleotide array)

The upgraded version of southern blotting technology is the microarray. This method was initially created to differentiate messenger RNA expression. It employed glass in place of nitrocellulose and nylon membrane as a support. Later, this method showed promise for the detection of viral pathogens without viral RNA amplification. The hundreds of unique probes that make up oligochips for oligonucleotide arrays are spotted onto a solid surface like a glass plate. With the virus removed from the plant, single stranded DNA hybridization probes with nucleotides ranging from 25 to 70 bases are used. The biggest limitation is cost because it needs a dust-free environment and a highly advanced processing unit for reading reactions and spotting probes and reading reactions and also needs dust-free room.

Chapter 10 ROLE OF MOLICULAR TECHNIQUES IN NEMATOLOGY

Ramavath Abhi, Shaik Munnysha, Manisha and Nitisha Gahlot

Abstract:

Nematodes that parasitize plants are a major issue for agriculture. They seriously harm agriculture all around the world. The output of the 40 most important food staples and commercial crops in the world is thought to be reduced by plant parasitic nematodes by roughly 12.5%. Furthermore, a sizable portion of the crop damage brought on by nematodes goes unreported. However, despite their influence on global agriculture, basic knowledge of plant parasitic nematodes' biology is lacking (Bird Mck. D., 1996). This can be explained by the challenges associated with gathering enough biological material for biochemical and molecular biology research. The majority of obligatory root parasitic nematodes in plants are tiny. Working with plant parasitic nematodes presents a number of challenges because of their challenging growth. Even when it is possible to gather enough material to conduct a biochemical experiment, the desire to repeat the experiment is thwarted by the need to wait until new material is accumulated once more. Nematicides must be applied to nematode-infested fields, crops must be rotated, and resistant plant cultivars must also be used to combat plant parasite nematodes. Researchers have only lately been able to answer questions that can be explained in terms of biochemistry and molecular biology thanks to the advent of new tools.

Keywords: Internal Transcribed Spacers, ribosomal DNA, Nematicide, Biochemistry

Introduction

Even for experts, it can be challenging to distinguish between different species of longidorid nematodes due to their highly preserved gross morphology. By their physical characteristics, the hosts they infect, their pathogenic impact on the host, and/or their geographic origin, individual nematodes are recognised and characterised. According to Taylor and Brown (2007), certain longidorid species are carriers of commercially significant plant viruses, making accurate identification of the species crucial. The tiny egg and larval stages frequently do not meet the required identification standards. The correct identification of a species may have significant effects on disease control, population biology, and taxonomy.

Molecular methods are thought to be especially helpful in cases when morphological characteristics provide ambiguity in interpretation. By having the ability to precisely amplify parasite DNA from small amounts of material, the polymerase chain reaction (PCR) specifically addresses the drawbacks of morphological identification. Furthermore, DNA is unaffected by the environment, by tissue, and by the stage of an organism's growth. Depending on the queries posed and the goal of the PCR, the target region for amplification by PCR is chosen. One of the

most often used target sites to distinguish between various nematode species is the nuclear ribosomal DNA (rDNA) cistron. (Zijlstra *et al.,* 1995; Powers *et al.,* 1997; Nadler *et al.,* 2000; Waeyenberge *et al.,* 2000; Lamberti *et al.,* 2001; Lamberti *et al.,* 2002; De Luca *et al.,* 2004a, 2004b).

Biochemical and molecular techniques in plant nematology:

- Crop protection and improvement have greatly benefited from the development of goods and procedures as a result of the use of biotechnology in agriculture.
- One example is the routine discovery and diagnosis of significant features in plants, microorganisms, and nematodes using molecular marker assisted techniques. Other examples include transgenic plants with resistance to insects, nematodes, herbicides, and quality traits like vitamin A.
- Due to the availability of a variety of biochemical and molecular approaches, all of these success stories are now both commercially viable and conceivable.
- The use of molecular methods to solve plant nematological issues is a relatively new development globally, and it is just getting started in India.The molecular techniques have added advantage under the following needs.

a) To understand plant nematode interactions in order to identify molecules important in designing novel nematicides or host resistance.
b) To decipher and analyze genetic variation within species and between species in some cases.
c) To isolate and study gene of interest conferring resistance from related and unrelated species where natural resistance is rare.

Molecular techniques:

1. Protein electrophoresis
2. Polymerase chain reaction (PCR)
3. PCR-RFLP (restriction fragment length polymorphism)
4. Multiplex PCR
5. Random amplified polymorphic DNA (RAPD)
6. Amplified fragment length polymorphism (AFLP)
7. Reverse dot hybridization
8. Sequencing of DNA
9. DNA bar-coding
10. Real-time PCR
11. Perspective of molecular diagnostics of nematodes

1. Protein electrophoresis:

History:

- Father of electrophoresis Arne Tiselius.
- The Nobel Prize in chemistry 1948.
- For his research on electrophoresis and adsorption analysis, especially for his discovering concerning the complex nature of the serum proteins.

What is Electrophoresis?

- A technique used in molecular biology and biochemistry to separate charged particles like proteins and nucleic acids
- An electric field is used to move negatively and positively charged molecules through a support medium.
- Positive or negative charge can be found on biomolecules including DNA, RNA, amino acids, and proteins, for example.
- Charged molecules gravitate toward the electrode with the opposite charge when these biomolecules are placed in an electric field because of the electrostatic attraction phenomena.
- Positively charged molecules gravitate toward the cathode, while negatively charged molecules gravitate toward the anode.
- The term "electroporesis" refers to the movement of charged particles while being affected by electricity.

Principle of Electrophoresis

- Any charged molecules or ion migrates when placed in an electric field.
- The rate of migration of a compound depends on its net charge. Size, shape, and applied current.
- This can be represented by following equation-

$$v = Eq/f$$

v = velocity of migration of molecule

E = Electric field in volt

f = frictional coefficient which function as mass and shape of molecules

q = net electric charge on molecule

The movement of charged molecule in an electric field is often expressed in term of electrophoretic mobility, which is defined as the velocity per unit of electric field.

- The first molecular method to be used in nematology was this one.
- On the basis of various molecular weights, soluble proteins isolated from nematodes are separated on polyacrylamide or starch gels under an electric field.

- Nematode extracts include a huge variety of proteins, however following thorough staining, particular band patterns can be identified for each sample.
- As taxonomic markers, differences in banding patterns across species or populations may be exploited.
- Isoelectric focusing (IEF), which divides proteins according to their charge in a pH gradient, makes proteins resolve into distinct bands and makes profiles more stable.
- Characterizing a single protein or a limited subset of proteins on gels using the enzyme-staining technique is another diagnostic tool.
- IEF is used as a standard diagnostic method for *Globodera pallida and G. restochiensis* as well as for the separation of other cyst nematode species.
- Extensive characterization of isozymes has been carried out for *Globodera, Heterodera, Radopholus, Meloidogyne, and Pratylenchus* and other nematode groups.
- For every given sample, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) offers a superior protein separation and fingerprint.
- Proteins are divided into two categories: mass-based categories in the second dimension and charge-based categories in the first. After staining, the position of individual proteins appears as spots of various size, shape and intensity.
- This technique has been applied to separate species and populations of *Globodera and Meloidogyne.*

2. Polymerase Chain Reaction (PCR):

What is PCR?

- PCR is a technique that takes specific sequence of DNA of small amount and amplifies it to be used for further testing.
- In vitro technique.

History of PCR

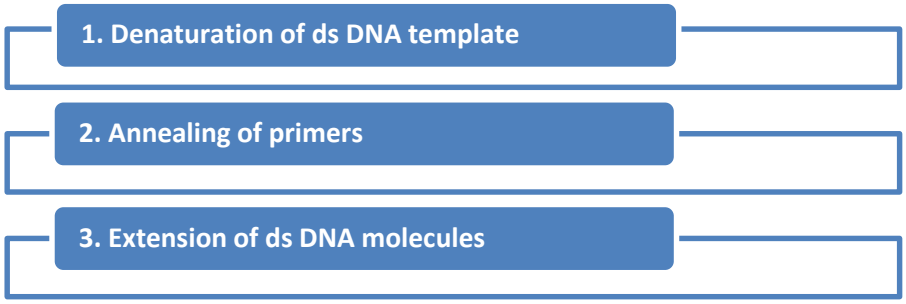
- 1983: Dr. Kary Mullis developed PCR
- 1985: first publication of PCR by Cetus Corporation appears in science
- 1986: Purified Taq polymerase is first used in PCR
- 1989: Science declares Taq polymerase ‘molecules of the year’
- 1993: Dr. Kary Mullis shares Nobel Prize in Chemistry for conceiving PCR technology.

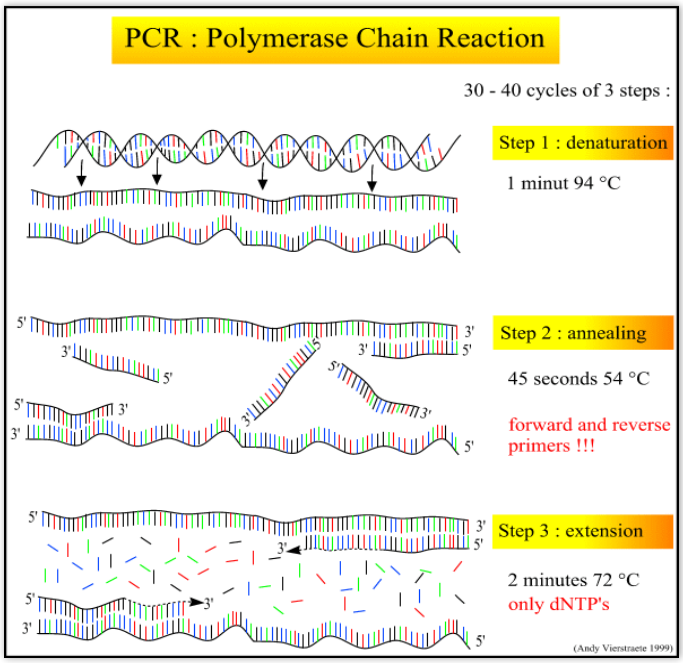
Principle of PCR

Purpose:

- To amplify a lot of double – stranded DNA molecules (fragments) with same (identical) size and sequence by enzymatic method and cycling condition.

Condition:





Chemical components of PCR

- Magnesium chloride: 0.5-2.5
- Buffer: Ph 8.3-8.8
- dNTPs: 20-200µM
- Primers: 0.1-0.5µM
- DNA Polymerase: 1-2.5 units
- Target DNA:≤ 1µg

Basic requirements for PCR reaction

- DNA sequence of target region must be known.
- Primers – typically 20-30 bases in size. These can be readily produced by commercial companies. Can also be prepared using a DNA synthesizer.
- Thermo- stable DNA polymerase – eg :Taq polymerase which is not inactivated by heating to 95 degree C
- DNA thermal cycle – machine which can be programmed to carry out heating and cooling of samples over a number of cycles.

Example of PCR programme

- Initial denaturation - 95C for 5 mins
- Thermo – cycle file - 30 cycles of
- Denaturation - 95C for 30 sec
- Annealing - 55C for 30 sec
- Extension - 72C for 45 sec
- Final extension - 72C for 5 mins
- Holding (soak) file usually 4C
- For researching the genetic variety of nematodes and identifying them, one of the most popular procedures is the PCR method.
- Using an enzyme catalyst, PCR is a quick, low-cost, and straightforward way to produce a substantial number of copies of DNA molecules.
- PCR is capable of amplifying and detecting any DNA fragment.
- The PCR procedure needs a DNA template with the target region on it, two oligonucleotide primers on either side of it, DNA polymerase, and four deoxynucleotide triphosphates (dAPT, dCTP, dGTP, and dTTP) combined in a solution containing magnesium ions (MgCl2).A primer is a short oligonucleotide, containing about two dozen nucleotides, which is complementary to the 3' end of each strand of the fragment that should be amplified.
- Primers anneal to the denatured DNA template and provide an initiation site for the elongation of the new DNA molecule.
- Universal primers are those complementary to a particular set of DNA for a wide range of organisms; primers matching only to certain species are called species-specific primers.
- The procedure consists of a succession of three steps determined by temperature conditions:
    - template denaturation (93 degree centigrade for 3-4 min);
    - primer annealing (55-60 degree centigrade for 1-2 min);
    - extension of the DNA chain (72 degree centigrade for 1-2 min).
- PCR is carried out for 30-40 cycles.
- As the result of PCR, a single target molecule of DNA is amplified into more than a billion copies.
- The resulting amplified products are electrophoretically separated according to their size on agarose or polyacrylamide gels and visualized using ethidium bromide, which interacts with double-stranded DNA and causes it to fluoresce under UV radiation.

- Once identified, nematode target DNA generated by PCR amplification can further be characterized by various analyses including restriction fragment length polymorphism (RFLP), dot blotting or sequencing.
- In some cases, the size of the PCR amplicon may serve as diagnostic marker for a nematode group or species.
- It has been shown that primers amplifying the control region of mitochondrial DNA (mtDNA) of root-knot nematodes generate different amplicon sizes for Meloidogyne javanica and M. arenaria; primers amplifying nuclear ribosomal intergenic spacer (IGS) generated species-specific size polymorphisms for *M. chitwoodi*, *M. hapla* and *M. fallax.*

3. PCR–Restriction Fragment Length Polymorphism (PCR–RFLP):

Principle of RFLP

- RFLP is an enzymatic procedure for separation and identification of desired fragments of DNA.
- Using restriction endonuclease enzymes fragments of DNA is obtained and desired fragments is detected by using restriction probes.
- May be used to differentiated two organism by analysis of patterns derived from cleavage of their DNA.

Pattern Generated Depends Mainly on:

- Differences in DNAs of selected strains
- Restriction enzymes used
- DNA probe employed for southern hybridization
- Point and frameshift mutations
- Differences in alleles for a particular sequence

Considerations for use of RFLP:

- Relatively slow process
- Use of radioisotopes has limited RFLP use to certified laboratories (but non-radioactive labelling systems are now in wide use)
- Co-dominant markers; often species-specific; highly locus-specific
- Specific to a single clone/restriction enzyme combination
- Need high quality DNA
- Need to develop polymorphic probes
- Expensive

Procedures or steps of RFLP test:

Step 1: Collection of sample:

DNA is extracted from the available sample.

Step 2: Restriction digest:

- The DNA in each sample is digest is digested with the same restriction enzymes.
- The enzymes RE has specific restriction site on the DNA, so it cut DNA into fragments.
- Different size of fragments are generated along with the specific desired fragments.

Step 3: Gel electrophoresis:

- The digested fragments are run in polyacrylamide gel electrophoresis or Agarose gel electrophoresis to separate the fragments on the basis of length or size or molecular weight.

Step 4: Denaturation:

- The gel is placed in sodium hydroxide (NaOH) solution for denaturation so that single stranded DNA are formed.

Step 5: Blotting:

- The single stranded DNA obtained are transferred into charge membrane i.e. nitrocellulose paper by the process called capillary blotting or electro-blotting.

Step 6: Baking and blocking:

- The nitrocellulose paper transferred with DNA is fixed by autoclaving.
- Then the membrane is blocked by using bovine serum albumin or casein to prevent binding of labeled probe nonspecifically to the charged membrane.

Step 7: Hybridization and visualization:

- The labeled RFLP probe is hybridization with DNA on the nitrocellulose paper.
- The RFLP probes are complimentary as well as labeled with radioactive isotopes so they form color band under visualization by autoradiography.

PCR-RFLP:

- Variation in sequences in PCR products can be revealed by restriction endonuclease digestion.
- The PCR product obtained from different species or populations can be digested by a restriction enzyme, after which the resulting fragments are separated by electrophoresis.
- If differences in fragment length occur within restriction sites, the digestion of the PCR products will yield RFLP, i.e. different RFLP profiles.
- PCR–RFLP of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene is a very reliable method for identification of many plant-parasitic nematode groups including cyst, root-knot, lesion and gall-forming nematodes, as well as nematodes from the genera *Bursaphelenchus* and *Aphelenchoides.*
- Using 6–9 restriction enzymes enables most of the economically important species of cyst nematodes to be distinguished from each other as well as from their sibling species.

- RFLP of the ITS-rDNA obtained after restriction with several enzymes and their combination identifies important root-knot nematode species; however, it fails to separate species from the tropical group, including *M. javanica, M. incognita and M. arenaria.*
- PCR–RFLP of the mtDNA fragment between the cytochrome oxidase subunit II gene and large subunit (LSU) has been applied successfully for diagnostics of these nematodes.

4. Multiplex PCR:

- Widespread molecular biology technique.
- Amplification of single template as well as multiple template in a single PCR experiment.
- By using multiple primer pairs in a reaction mixture.

Advantages:

- This technique has the potential to produce considerable savings in time and effort within the laboratory.
- Without compromising on the utility of the experiment.

Disadvantages:

- Optimization is difficult since many sets of forward and reverse primers are to be designed for use.
- Increases cost.
- Presence of multiple primer may lead to cross hybridization with each other and the possibility of mis- priming with other templates.

Types of Multiplex PCR

1. Single template PCR reaction:

- This technique uses a single template which can be a genomic DNA.
- Along with several pairs of forward and reverse primers to amplify specific regions within a template.

2. Multiple template PCR reaction:

- This technique uses multiple template
- Several primer sets of forward and reverse primers for each template and regions within the template; in the same reaction tube.
- This type of PCR constitutes a major development in DNA diagnostics and enables the detection of one or several species in a nematode mixture by a single PCR test, decreasing diagnostic time and costs.
- In multiplex PCR, two or more unique targets of DNA sequences in the same sample are amplified by different primer pairs in the same amplification reaction.
- Multiplex PCR for detection of a single nematode species uses two sets of primers:

(i) one set is to amplify an internal control (e.g. universal primers for D2–D3 expansion regions of the 28S rRNA gene) confirming the presence of DNA in the sample and the success of PCR.

(ii) the second set, including at least one species-specific primer, is targeted to nematode DNA sequences of interest .

- Diagnostics using multiplex PCR with species-specific primers have been developed for a wide range of plant-parasitic nematodes: *G. pallida, G. rostochiensis, Heterodera schachtii, H. glycines, Ditylenchus dipsaci* and species of *Meloidogyne and Pratylenchus.*

5. Random Amplified Polymorphic DNA (RAPD):

Introduction:

- RAPD markers are decamer DNA fragments.
- RAPD is a type of PCR reaction, segments amplified are Random.
- No knowledge of DNA sequence required. Hence a popular method.
- In recent years, RAPD is used to characterize, and trace, the phylogency of diverse plant and animal species.
- Identical 10 – mer primer will or will not amplify a segment of DNA, depending on positions that are complementary to the primer sequence.

How it works?

- The principle is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template.
- This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome.
- These amplified products (of up to 3 kb) are usually separated on agarose gels (1.5-2.0%) and visualized by ethidium bromide staining.
- Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites.
- Recently, sequence characterized amplified regions (scars) analysis of rapd polymorphisms showed that one cause of rapd polymorphisms is chromosomal rearrangements such as insertions/deletions.
- In order for PCR to occur:

1. The primer must anneal in a particular orientation (such that they point towards each other)
2. They must anneal within a reasonable distance of one another.

RAPD involve following steps:

1. The DNA of a selected species is isolated

- Quality influences the outcome of the PCR
- High molecular weight

- No impure template
- Less amount of RNA

2. An excess of selected decaoligonucleotide added

- 10 base primer for PCR, only 1 primer per reaction
- Short primer bind randomly on the chromosome

3. This mixture is kept in a PCR equipment and is subjected to repeated cycles of DNA denaturation- renaturation-DNA repliction
4. During this process, the decaoligonucleotide will pair with the homologous sequence present at different locations in the DNA
5. DNA replication extendthe decaoligonucleotide and copy the sequence continuous with the sequence with which the selected oligonucleotide has paired
6. The repeated cycles of denaturation-renaturation DNA replication will amplify this sequence of DNA
7. Amplification will takes place only of those regions of the genome that has the sequence complementary to the decaoligonucleotide at their both ends
8. After several cycles of amplification the DNA is subjected to gel electrophoresis
    - Initial denaturation at 94˚ C for 10min
    - Denaturation at 94˚ C for 1min
    - Annealing at 37-45˚ C for 1min
    - Extension at 72˚ C for 1min
    - Final extension at 72˚ C for 10min
    - Cooling at 4˚ C
9. The amplified DNA will form a distinct band. It is detected by ethidium bromide staining and visible fluorescence's under U.V. light
    - This method uses a single random primer of about ten nucleotides long for creating genomic fingerprints.
    - This technique is often used for estimating genetic diversity between individuals, populations or closely related species.
    - In this PCR approach, the short primer anneals to numerous similar sequences within the genome during the annealing step of the PCR cycle, which occurs at a lower temperature than does 'classical' PCR.
    - If two complementary sequences are present on opposite strands of a genomic region in the correct orientation and within a close enough distance from each other, the DNA fragment between them can be amplified by PCR.
    - Amplified DNA fragments obtained using different random primers from different samples are separated on gels and compared.

- RAPD polymorphisms result from the fact that if a primer hybridization site in a genome differs by even a single nucleotide, the change can lead to elimination of a specific amplification product.
- The resulting individual bands are considered as equivalent independent characters.
- The band polymorphism can be binary scored and the data matrix is used for calculating the genetic distance between the samples under study and then presented as a dendrogram.
- Reproducibility of results is the most critical point for application of this technique.
- The RAPD technique has been widely applied for separation of closely related species and studies of intraspecific variability of *G. pallida, H. glycines, Radopholus similis, D. dipsaci* and many other species.
- Specific sequences for certain species or races, called sequence-characterized amplified regions (SCARs) can be derived from RAPD fragments.
- Specific pairs of SCAR primers have been designed for identification of M. chitwoodi, M. fallax, M. hapla and other root-knot nematode species as well as identification of the normal and the giant populations of the D. dipsaci complex.

6. Amplified fragment length polymorphism (AFLP):

- The AFLP technique is used to visualize hundreds of amplified DNA restriction fragments simultaneously.
- AFLP technology combines the power of restriction fragments length polymorphism (RFLP) with the flexibility of PCR- based technology by ligating primer- recognition sequences to the restricted DNA.
- Restriction endonuclease digestion of DNA
- Ligation of adaptors
- Amplification of ligated fragments
- Separation of the amplified fragments via electrophoresis and visualization
- AFLPs have stable amplified and good repearability
- Advantages of the AFLP:
- Only small amounts of DNA are needed..
- Unlike randomly amplified polymorphic DNAs (RAPDs) that use multiple, arbitrary primers and lead to unreliable results, the AFLP technique uses only two primers and gives reproducible results.
- Many restriction fragments subsets can be amplified by changing the nucleotide extensions on the adaptors sequences. Hundreds of markers can be generated reliably.
- High resolution is obtained because of the stringent PCR conditions.
- The AFLP technique works on a variety of genomic DNA samples.

- No prior knowledge of the genomic sequence is required.
- The technique is one of the most popular fingerprinting techniques.
- AFLP is also a random amplification technique, which does not require prior sequence information.
- AFLP produces a higher number of bands than is obtained by RAPD.
- It is a much more reliable and robust technique, unaffected by small variations in amplification parameters; however, it is more expensive.
- The AFLP technique represents a conceptual and practical advance in DNA fingerprinting. It comprises the following steps:

(i) restriction of the total DNA with two restriction enzymes;
(ii) ligation of double-stranded adapters to the ends of the restriction fragments;
(iii) amplification of a subset of the restriction fragments using two 17–21 nucleotide primers complementary to the adapter and one that is 1–3 nucleotides adjacent to the restriction sites;
(iv) separation and visualization of the AFLP–PCR fragments with a variety of techniques, usually on denaturing polyacrylamide gels with further staining.

- A comparative study of *Globodera* species and populations using AFLP revealed greater inter- and intraspecific variability than obtained by RAPD, and enabled subspecies of *G. tabacum* to be distinguished.
- AFLP analysis also showed a clear distinction between normal and the giant populations of the *D. dipsaci* complex.

7. Reverse dot blot hybridization

- This technique involves the use of PCR simultaneously to amplify and label target DNA to generate digoxigenin dUTP-labelled amplicons that are hybridized to the specific, immobilized oligonucleotide probes on a membrane.
- This approach can be used for simultaneously identifying many different nematode species from a single sample.
- The presence of particular species in the sample is determined from the positive reactions (dark dot) in the test.
- A reverse dot blot assay has been developed for identification of several *Pratylenchus* species using oligonucleotides designed from the sequences of the ITS region of rRNA.

8. Sequencing of DNA:

Introduction:

- The information content of DNA is encoded in the form of four bases (A, G, C and T) and the process of determining sequence of these bases in a given DNA molecule is referred to as DNA sequencing.

- DNA fragments can be analyzed to determine the nucleotide sequence of DNA and to determine the distribution and location of restriction sites.

Fundamental reasons for knowing the sequence of DNA molecule:

- To characterize the newly cloned DNA.
- For predictions about its functions.
- To facilitate manipulation of the molecules.
- To confirm the identity of a clone or a mutation.
- To check the fidelity of newly created mutation and ligation junction.
- Screening tool to identify polymorphisms and mutation in genes of particular interest.
- To confirm the product of a PCR.
- The process of determining the order of the nucleotide bases along a DNA strand is called sequencing.
- Two different procedures have been developed for DNA sequencing, i.e. the chemical degradation (Maxam–Gilbert) method and the chain termination (Sanger dideoxy) method, the latter being more commonly used.
- The chain termination sequencing method is similar to PCR in that it involves the synthesis of new strands of DNA complementary to a single-stranded template.
- The sequencing reaction components are template DNA, DNA polymerase with reaction buffer, one primer and the mixture of all four deoxynucleotide (dNTP) and four dideoxynucleotide (ddNTP) labels, each with a different coloured fluorescent dye.
- As all the four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a ddNTP.
- As the dideoxy sugar lacks a 3′-hydroxyl group, continued lengthening of the nucleotide chain cannot occur.
- Thus, the ddNTP acts analogously to a specific chain-terminator reagent.
- Therefore, the result is a set of new chains with different lengths.
- These fragments are then separated by size using electrophoresis.
- As each labelled fragment reaches the bottom of the gel, a laser excites the fluorescent molecule, which emits light of a distinct colour.
- A detector records the colour of fluorescence of each band.
- A computer program Chromas can display sequence chromatogram from files.

9. DNA bar-coding:

What is DNA bar coding?

- DNA barcoding is a standardized approach to identifying plant and animals by minimal sequences of DNA, called DNA barcodes.

- DNA barcodes – short gene sequences taken form a standardized portion of the genome that is used to identify species.
    - Founder of DNA barcoding – Dr. Paul D. N. Hebert.
- The bar-coding technique is a promising tool for nematode identification.
- It is based on the idea that a particular nucleotide sequence from a common gene can serve as a unique identifier for every species, and a single piece of DNA can identify all life forms on earth.
- Molecular bar-coding involves isolation of the nematodes (as individuals or in bulk), amplification of the target gene, cloning, sequencing and phylogenetic analysis leading to the assessments of species content, abundance and diversity.
- A molecular bar-code derived from single specimen PCR and sequencing of the 5′ segment of the 18S rRNA gene for the estimation of nematode diversity in Scottish grassland.
- Currently, there is insufficient information in databases for extensive nematode species identification based on DNA fragments.
- However, the increasing deposition of DNA sequences in GenBank and NemATOL databases will be beneficial for diagnostics.

10. Real-time PCR:

What is Real - Time PCR?

- Real – Time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reaction over a range of cycles.

Introduction:

- Real time PCR is a advanced biotechnological instrument. The term real time denotes it can monitor the progress of the amplification when the process is going on.

Working procedure of Real – Time PCR :

1. **Amplification:**

Detection:

- The detection is based on fluorescence technology.
- The specimen is first kept in proper well and subjected to thermal cycle like normal PCR but at this machine it is subjected to tungsten or halogen source that lead to fluorescence the marker added to the sample and the signal is amplified with the amplification of copy number of sample DNA.
- The emitted signal is detected by a detector and sent to computer after conversion into digital signal that is displayed on screen.
- The signal can be detected when it comes up the threshold level, this cause elimination of background noise.

10. Real-time PCR:

- DNA technology also provides several methods for quantification of nematodes in samples.
- Real-time PCR requires an instrumentation platform that consists of a thermal cycler, optics for fluorescence excitation as well as emission collection, and computerized data acquisition and analysis software.
- The PCR quantification technique measures the number of nematodes indirectly by assuming that the number of target DNA copies in the sample is proportional to the number of targeted nematodes.
- Most of the difficulties with the PCR technique arise because only a very small number of the cycles (4–5 out of 40) contain useful information.
- The early cycles have an undetectable amount of DNA product; the final cycles, or the so-called plateau phase, are almost as uninformative.
- Quantitative information in a PCR comes from those few cycles where the amount of DNA grows logarithmically from just above background to the plateau.
- The real-time technique allows continuous monitoring of the sample during PCR using hybridization probes (TaqMan, Molecular Beacons and Scorpions), allowing simultaneous quantification of several nematode species in one sample, or double-stranded dyes such as SYBR Green, and providing the simplest and most economical format for detection and quantification of PCR products in real-time reactions.
- Compared with traditional PCR methods, real-time PCR has advantages.
- It allows for faster, simultaneous detection and quantification of target DNA.
- The automated system overcomes the laborious process of estimating the quantity of the PCR product after gel electrophoresis.

- Real time has been used for detection and quantification *of Paratrichodorus pachydermus, H. schachtii, G. pallida and D. dipsaci* as well as for estimation of the number of virus-vectoring *trichodorid* nematodes.

Advantages:

- Real Time PCR has many advantages over normal PCR:
- It does not require gel preparation like traditional PCR.
- It is not time consuming like normal PCR.
- Less complexity at the quantification of sample.

11. Perspectives of molecular diagnostics of nematodes:

- Achieving simpler, cheaper and more reliable DNA diagnostics may be realized by DNA-chip technology.
- DNA-chip analysis is a technology that is still being shaped; a number of different technical methods are being used.
- A DNA chip is a small piece of silicon glass (~1 cm^2) to which a large number of specific synthetic, single-stranded DNA oligonucleotides or probes have been chemically bound using a high-speed robotic instrument.
- The individual DNAs from a heterogeneous sample are labelled with dyes.
- Then labelled DNA is inserted into the chip and allowed to hybridize with probes.
- DNA probes anneal selectively only to those DNA molecules whose nucleotide sequences are exactly complementary.
- A computer reads the pattern of annealing and reports which species are present in a sample.
- Detection of hybridization on a DNA chip can be done through mass spectrometry, avoiding the expense and time required for fluorescence labelling.

Genes used for Molecular Systematics:

1. Nuclear ribosomal RNA genes.
2. Nuclear protein-coding genes.
3. Mitochondrial DNA

1. Nuclear ribosomal RNA genes:

- Historically, the only nuclear genes with a high enough copy number for easy study were ribosomal genes.
- These genes code rRNAs , which are nearly two-thirds of the mass of the ribosome.
- The genes encoding rRNA are arranged in tandem, in several hundred copies, and are organized in a cluster that includes a small subunit (SSU or 18S) and a large subunit (LSU or 26–28S) gene, which are themselves separated by a small 5.8S gene.
- The whole set of genes are transcribed as a single unit.

- There are 100–150 copies of the rRNA gene on chromosome I in *C. elegans*, whereas *C. briggsae* has 55 copies.

In addition to these coding sequences, the rDNA array also contains spacer sequences, which contain the signals needed to process the rRNA transcript: an external transcribed spacer (ETS) and two ITSs, ITS1 and ITS2.

- A group of genes and spacer sequences together make up an rRNA transcript unit.
- These units are separated from each other by an intergenic spacer (IGS), also known as a non-transcribed spacer (NTS).
- The rRNA (18S and 28S) genes evolve slowly and can be used to compare distant taxa that diverged a long time ago, whereas external and intergenic spacers have higher evolution rates and so have been used for reconstructing relatively recent evolutionary events and for the comparison of closely related species and subspecies.
- The IGS region contains many repeats and is more variable than the ITS region.
- For example, the detailed sequence analyses of the ITS region for the rootknot nematodes from tropical group revealed extremely high diversity, even within individual nematodes.
- This ITS diversity was broadly structured into two very different groups that are 12–18% divergent: one with low diversity (less than 1%) and one with high diversity (6–7%), which could be explained by the presence of pseudogenes or non-homogenized paralogous copies as the result of ancient hybridization events.

2. Nuclear protein-coding genes:

- Protein-coding genes have some advantages over rRNA genes and their spacers in that the alignment of sequences is less problematic.
- Protein sequences also lend themselves to different phylogenetic weighting of bases by codon position.
- The intron position patterns may also serve as decisive markers for phylogenetic analysis.
- RNA polymerase II, actin, major sperm protein, heat-shock proteins and other genes have been used for phylogenetic studies of *tylenchds, pratylenchids,* cyst and other nematode groups.
- In most cases, nematode phylogenies reconstructed from these data-sets do not differ from those constructed from nuclear ribosomal genes or spacers.

3. Mitochondrial DNA:

- Mitochondrial DNA (mtDNA) has been used to examine population structure and evolutionary relationships between different nematode groups.
- All nematode mtDNAs are circular, double-stranded DNA molecules.
- The mitochondrial genome of the majority of nematodes includes:

- 12 protein-coding genes, all components of the oxidative phosphorylation system including subunits of cytochrome c oxidase (COI–COIII);
- 22 transfer RNA (tRNA) genes;
- rRNA genes encoding SSU and LSU rRNAs.

- In addition, there is usually a non-coding AT-rich region, or a region with high levels of the nucleotides adenine and thymine in the mitochondrial genome containing an initiation site for replication and transcription.
- The arrangement of genes in the mitochondrial genome is not consistent within Nematoda.
- Nematodes are characterized by a surprising variation in gene order.
- A unique feature of the mitochondrial genome organization of nematodes is that some of them, for example, *G. pallida*, may contain a population of circular mtDNA of varying sizes (~6.3–9.5 kb) and gene contents.

Resistant gene cloned against various nematodes:

Sr. No.	Name of gene	Host from which isolated	Against nematode
1.	*Hero-A*	Tomato *(Lycopersicon peruvianum)*	*Globodera rostochiensis, G. pallida*
2.	*Hmg2*	Tomato *(Lycopersicon peruvianum)*	*Meloidogyne incognita, M. hapla*
3.	*Mi-1,Mi-2,Mi-3*	Tomato (*Lycopersicon peruvianum)*	*M. incognita, M. arenaria, M. javanica*
4.	*Mia-1, Mia-2, Mia-3, Mia-4*	Alfalfa	*M. incognita*
5.	*Hs1 pro-1*	Sugarbeet (*Beeta procubance*)	*Heterodera schachtii*
6.	*CpTi*	Cowpea	*Globodera pallida*
7.	*Cre-1, Cre-2, Cre-3*	Wheat	*H. avenae*
8.	*Gro-1*	Potato (*Solanum tuberosum ssp. andigena)*	*G. rostochiensis*
9.	*Gpa-1,Gpa-2*	Potato (*Solanum tuberosum ssp. andigena*)	*G. rostochiensis*
10.	*Me-1,Me-3*	Capsicum (*Capsicum annuum)*	*M. incognita*
11.	*Rk*	Cowpea	*M. incognita*
12.	*Rhg-4*	Soyabean	*Heterodera glycine*
13.	*Ma*	Myrobalan plum *(Prunus cerasifera*)	*M. enterolobii*

Nematode genome size:

Sr. No.	Nematode	Genome size	Number of protein coding genes
1.	*Haemonchus contortus*	53mb	---
2.	*Caenorhabditis elegans*	100mb	25,244
3.	*Caenorhabditis briggsae*	104mb	21,986
4.	*Caenorhabditis angaria*	80mb	26,265
5.	*Pristionchus pacifiicus*	100mb	24,217
6.	*Meloidogyne hapla*	62mb	13,072
7.	*Meloidogyne incognita*	82mb	21,232
8.	*Aphelenchus avenae*	34mb	---
9.	*Heterodera glycines*	123mb	29,769
10.	*Heterodera maritima*	36mb	---
11.	*Globodera pallida*	124mb	14,580
12.	*Globodera rostochiensis*	96mb	13,083
13.	*Pratylenchus coffeae*	18mb	---
14.	*Tylenchorhynchus graciliformis*	43mb	---
15.	*Merlinius brevidens*	32mb	---
16.	*Longidorus kuiperi*	56mb	---
17.	*Bursaphelenchus xylophilus*	75mb	18,074
18.	*Brugia malayi*	85-95mb	21,332
19.	*Trichinella spiralis*	240mb	16,380
20.	*Ascaris suum*	273mb	18,542

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