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BIOCHEMICAL AND MOLECULAR ASPECTS OF DISEASE RESISTANCE IN PLANTS

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ABSTRACT

It is well known that plant pathogenic fungi and bacteria inflict enormous crop losses. Genetic basis of disease resistance in crop plants and virulence in pathogens are reasonably well understood. In the quest to elucidate the underlying mechanisms of plant disease resistance, biochemical aspects were studied and this, in turn, have led to an understanding of molecular genetic basis of resistance also in some crop plants. This article reviews the available literature on the biochemical and molecular aspects of plant disease resistance and indicates possibilities for future research to evolve genetically engineered crop plants that can dissuade the pathogen at the earliest stage in infection, the recognition step leading to the signal transduction.

INTRODUCTION

Plant diseases caused by viral, bacterial or fungal pathogens are the causes of enormous crop losses. The most important pathogens of economically important crop plants are biotrophic and necrotrophic fungi. Under natural conditions plants co-exist with a variety of microorganisms which include the pathogenic fungi. Three possible relationships of plants with the co-existing microbes are: (a) neutral or non-interaction, (b) beneficial interaction, and (c) harmful interaction. Out of the three, the third relationship causes disease and damage to the host plants. This is conditioned by the ability of the pathogen to recognize and colonize the host plant and the ability of the plant to counteract or otherwise of the pathogen's activities. Final outcome of these interactions is controlled by the genetic constitution of the host and the pathogen as well as environment to some extent. A plant is said to be resistant if the pathogen is unable to colonize and spread within the host plant tissues, whereas in a susceptible plant the pathogen can spread systemically in the host tissues causing widespread damage. These interactions are termed as incompatible and compatible, respectively. Analyses of these interactions by classical genetic techniques have led to such proposals as the gene-for-gene hypothesis (Flor, 1956). However, these proposals and related studies contributed little to the enhancement of our understanding of the actual mechanism of disease resistance or susceptibility. With the increasing application of molecular techniques in host-pathogen interactions (Kerr, 1987; Leong & Holden, 1989), it may soon be possible to elucidate the mechanisms of disease resistance. This possibility draws considerable support from the recent literature reviewed below.

As plants are sedentary organisms, they are vulnerable to adverse environmental conditions as well as biotic adversaries like pathogens and plant eating insects. Capabilities of plants to dissuade the adversaries depend a great deal on the preformed structures and pathogen induced responses. These are called passive and active defence, respectively.

(a) Passive Defence Mechanisms

Plants possess waxy cuticles covering the leaves and other parts. The major chemical constituent of cuticle is cutin, an insoluble polyester of C16 and C18 fatty acids (Kolattukudy, 1985). This protects the plant parts from desiccation as well as from the invasion of microoganisms. Pathogens enter plant tissues either through wounds or by secreting degradative enzymes such as cutinases, which help in the penetration of hyphae through cuticle.

(i) *Cellulose and lignin :* Cells of higher plants also possess cellulosic walls. Cellulose, a β-1, 4 glucan polymer constitutes 20-30% of primary cell wall material (McNeil et al., 1984). Additionally, proteins and other polysaccharides also contribute to the structure and function of plant cell walls and play a significant role in defence. Pathogens have to secrete cell wall degrading enzymes to gain entry into plant tissues. In the process, they trigger a variety of plant defence responses. The degraded products from host and/or pathogen cell walls are known to act as elicitors of these responses (Darvill & Albersheim, 1984; Ryan, 1987). Disruption of the host cell wall and cell itself results in the release of substances, which can be toxic to the pathogens. Pathogen induced lignification of cell walls also comprises a defence response manifested in the hypersensitivity of plants to pathogens. Lignin is a random condensation product of phenylpropanoid alcohols and is a component of secondary cell walls. Lignification as a defence response is triggered by the activity of many genes specifying lignin biosynthetic enzymes such as cinnamyl alcohol dehydrogenase, polyphenol oxidase and peroxidases (Carr & Klessig, 1989).

(ii) *Callose :* As fungi begin to penetrate the cell wall, either with infectious hypae or haustoria, the resistant host responds by synthesizing more of cellulose and callose (β-1, 3 glucan) which are added to the inside of the cell wall. These appositions may result in the formation of dome-shaped or elongated papillae. Cells adjoining those invaded by pathogen also may deposit new carbohydrates onto the thickening secondary walls. In susceptible plants, secondary wall thickening and papillae are either absent or poorly developed (Aist, 1976; Hohl & Stossel, 1976).

(iii) *Cell wall proteins :* Proteins associated with cell walls are : hydroxyproline rich glycoprotein (HRGP), glycine rich proteins (GRP) and leaf specific thionins which have antifungal activity and constitute the first line of defence against pathogens. The name *extensin* was proposed for the insoluble hydroxyproline rich glycoprotein of primary cell walls. The most remarkable property of this protein is its insolubility. It can not be extracted from the cell walls with any of the conventional protein solvents, but by mildly acidified $NaClO_2$. Extensin is a basic protein (Muray & Northcote, 1978; Stuart & Varner, 1986), whose biological function has been the subject of extenisve speculation. Two functions have been proposed: (a) control of cell expansion, and (b) resistance to invading pathogens (Fry, 1982). This protein is known to accumulate in response to pathogen invasion and wounding (Bowles, 1990). Extensin polypeptides are noted to be encoded by a multi gene family, although alternative splicing of mRNA's encoded by a

single gene is also possible. Extensin, by virtue of its tight cross linking renders the wall indigestible by invading pathogens (Fincher et al., 1983). It is highly resistant to proteases and could only be hydrolysed by trypsin, if the conjugated arabinose residues are removed (McNeil et al., 1984).

The glycine-rich proteins are characterised by a high glycine content (>60%), which are isolated from a variety of plant sources (Varner & Cassab, 1986; Reddy & Poovaiah, 1987). This protein is known to increase rapidly in tissues in response to wounding (Bowles, 1990).

Pathogen induced changes in cell wall structure leading to lignification, apposition of cellulose/callose and HRGP/GRP can be considered as a part of passive as well as active defence mechanisms as these processes strengthen the basically passive barriers to pathogen penetration but in response to the presence of the pathogen (Chakravorty & Scott, 1991).

(b) Active Defense Mechanisms

As already mentioned, to gain entry into the host plant and spread, pathogens secrete cell wall degrading enzymes. During this process, they inadvertently trigger off a number of other signals, which activate a variety of plant defence responses. These signal-response processes are collectively termed as active defence mechanisms and are briefly reviewed below.

(i) *Phytoalexins :* Phytoalexins are low molecular weight compounds with broad-spectrum antibiotic activity and are believed to play an important role in arresting the growth of fungal pathogens in resistant plants. These are products of plant secondary metabolism. Their production is known to require the induction of enzymes catalyzing the reactions of phenyl propanoid biosynthesis (Bryngelsson & Collinge, 1991). The expression of genes responsible for the activation of phenyl propanoid pathway could also be induced by elicitors which are partial breakdown products of fungal and/or plant cell walls (Tepper & Anderson, 1986). Other biotic agents like glucans, proteins, glycoproteins and fatty acid derivatives also elicit induction of phytoalexin synthesis. Abiotic elements known to induce the enzymes of phytoalexin biosynthesis include mechanical injury, ultraviolet radiation and heavy metals. Whatever the elicitors, the induction of these genes is very rapid and also linked to the expression of genes specifying HRGP precursors and the enzymes for lignin biosynthesis (Car & Klessig, 1989).

In pheyl propanoid biosynthesis, phenylalanine ammonia lyase (PAL) is reported to be the enzyme cataylzing the first step: deamination of phenylalanine to yield transcinnamic acid. Cinnamic acid obtained in this step is the precursor of all phenyl propanoids. PAL has been reported to be induced in response to the infection by powdery mildew fungus in barley (Shiraishi et al., 1989). In soybeans PAL is the first enzyme in the biosynthetic pathway of pterocarpan phytoalexins. Inhibition of this enzyme by α-2-amino-3-phenyl propionic acid in soybeans leads to the loss of resistance to *Phytophthora megasperma* f.sp. *glycinea*. In parsley, a small family of at least 4 genes encodes PAL. PAL accumulated around infection sites following fungal inoculation in parsley seedlings (Bowles, 1990). Several other enzymes of phytoalexin biosynthetic pathways have been studied at the molecular level. These are: 4-coumarate CoA ligase, 6-hydroxychalocone synthetase, chalocone isomerase and cinnamic acid 4-hydroxylase and their induction in most cases has been shown to be at the transcriptional level (Carr & Klessig, 1989).

Sorghum accumulated a complex of phenols in response to fungal infection and notable among them are fungitoxic compouds

apigenidin and leuteolinidin, the 3-deoxyanthocyanidins. These were considered phytoalexins due to their fungitoxicity and they accumulated repidly in mesocotyles and leaves of sorghum following infection.

Another interesting phytoalexin of 3-deoxyanthoayanidin group that was found in sorghum leaves infected with *Colletotrichum graminicola* is a caffeic acid ester of arabionsyl-5-apigenidin.

In host-fungal pathogen interactions, it was proposed that the inability of the fungus to detoxify phytoalexins constitutes the resistance of the host plant (Van Etten et al., 1989). Some fungi possess mechanisms of phytoalexin tolerance, which does not involve metabolic degradation. The bean pathogen, *Fusarium solani* f.sp. *phaseoli* can detoxify at least four major phytoalexins of bean namely rievitone, phaseolin, phaseollidin and phaseollinisoflavone and the mechanisms of inactivation are elucidated (Van Etten et al., 1989). Lubinin and rishitin are two major sesquiterpenoid phytoalexins of potato. Although pathogenicity was often linked to phytoalexin detoxification and expression of concerned genes, there are fungi which are known to be sensitive to phytoalexins even though they can metabolize them (Van Etten et al., 1982).

In essence, the phytoalexin defence can be considered as the protection offered by the phenyl propanoid and lignin biosynthetic enzyme to the plants against pathogens.

(ii) *Proteinase inhibitors :* The existence of enzyme inhibiting proteins was first discovered by Weinland (1903)* who used the term "Anti enzymes" to explain the resistance of certain nematodes to the enzymes of alimentary canal. Induction, synthesis and accumulation of proteinase inhibitors in plants is triggered by a proteinase inhibitor-inducing factor (PIIF). This is large pectin fragment (200 kDa) released from plant cell walls. Proteinase inhibitors are low molecular weight proteins and possess the capacity to form stoichiometric complexes with various enzymes resulting in the competitive inhibition of their catalytic properties (Ryan, 1987).

*Not seen in original, cited from Kumari (1993).

The occurrence of proteinase inhibitors in plant storage organs is widespread (Vogel et al., 1966). They were isolated mainly from Leguminosae, Solanaceae and Gramineae families (Richardson, 1981). Two inhibitors from soybean were studied extensively: the Kunitz inhibitor (21.5 kDa) which inhibited the activity of trypsin and the Bouman-Birk inhibitor (8 kDa) which inhibited the activities of both trypsin and λ-Chymotrypsin (Kunitz, 1947; Birk, 1985; Odani & Ikenaka, 1972).

Inhibitors contain active sites for the inhibition of proteolytic enzymes that endow them with their specificity. Trypsin-specific inhibitors always have a lys-x or arg-x sequence at their binding site, whereas chymotrypsin-specific inhibitors usually carry a leu-x motif at their active centres (Birk, 1974).

The possible role of proteinases in facilitating microbial invasion of plant tissues is well-documented (Ryan & Shumway, 1971). It is possible that proteinase inhibitors in tissues are directed against the microbial extracellular proteinases and severely retard proteolysis of cell walls and membrane proteins consequently reducing disruption of cell organisation. Most of the proteolytic enzymes known to be secreted by microorganisms are trypsin-like or chymo-trypsin-like in their specificities (Vogel et al., 1966) and are strongly inhibited when challenged with trypsin/chymotrypsin inhibitors. Thus, the presence of inhibitors in plant tissues could be an asset to the plant in arresting the extracellular proteinases of pathogens. The emerging picture from structural and specificity similarities of plant

inhibitors from diverse sources indicates that inhibitory capacity is essential for survival. Recent advances in physiology of inhibitors in plants suggested that they may have important roles as: (i) storage proteins, (ii) regulatory proteins, and (iii) protective agents against microbial proteins. The storage role for the inhibitors was inferred by their presence in abundance in storage parts like seeds and tubers.

In barley, inhibitors of *Aspergillus* proteinase appear to be present in all stages of development in meristematic tissues but most predominant in the developing endosperm (Kirsi & Mikola, 1971, 1977). In contrast to trypsin inhibitors, this proteinase inhibitor is synthesized in the late stage of grain development (Kirsi, 1973). In tomato also the inhibitor levels increased following infection by *Phytophthora infestans*. This response was observed only in resistant varieties (Peng & Black, 1976). Wound damage to the leaves of plants is known to result in the induction of synthesis of proteinase inhibitor proteins at the wound sites as well as in distal leaves (Green & Ryan, 1972; Brown & Ryan, 1984).

(iii) *Lectins :* Lectins are carbohydrate-binding proteins that bind glucans of glycoproteins, glycolipids or polysaccharides with a high affinity. These are thought to play a defence role as they have the potential to bind to fungal or bacterial cell walls (Chrispeels & Raikhel, 1991). Accumulation of lectins specifically in the embryo is an indication of possible protective role against pests and pathogens. The chitin binding *Datura* seed lectins are extracellular and offer protection to the plant and wheat germ agglutinin is also a lectin, which interacts with chitin oligomers. Besides the above, nettle lectin also carries a chitin binding domain.

(iv) *Peroxidases :* A substantial raise in the activity of peroxidase enzymes during infection, their extracellular location and their role in lignification and suberization as well as in the polymerization of HRGPs suggest a function for them in defence against diseases based on cell wall modification (Bryngelsson & Collinge, 1991). There are many isozymes of peroxidases, some of which are highly acidic, while others basic. However, all of them are glycosylated Hemo-proteins of 30-35 kDa molecular weight. Some peroxidase isozymes are known to possess phenol oxidase activity also (De Biasi & Badiani, 1990). Peroxidase activity increases following fungal infection in many plants (Hislop & Stahmann, 1971; Arora & Bajaj, 1985).

(c) Inhibitors of Protein Synthesis

Two groups of protein synthesis inhibitors are reported from the endosperm of several cereals. Ribosome inactivating proteins (RIPs) comprise the first group and the second group comprises of a family of sulphur containing low molecular weight proteins known as thionins or purothionins.

(i) *Ribosome inactivating proteins :* Ribosome inactivating proteins found in the seed extracts are known to prevent protein synthesis, widely distributed in the plant kingdom and act on the large ribosomal sub-unit to inactivate the ribosome to carry out the polypeptide chain elongation (Jimenez & Vazquez, 1985; Merino et al., 1990; Stripe & Hughes, 1989). Single chain proteins with ribosome inactivating properties are termed type-I wherease double chain RIPs that combine the properties of RIP and lectin are called type II. RIPs do not inactivate 'self' ribosomes but show varying specificities towards ribosomes of different distantly related species and fungi (Roberts & Selitrennikoff, 1986). RIPs are being used as immunotoxins, antiviral and antifungal agents (Endo et al., 1987, 1988; Leah et al., 1991).

(ii) *Purothionins :* These are a family of low molecular weight, sulphur containing basic

proteins of widespread occurrence in plant kingdom (Bohlman et al., 1988). The thionins of barley are well characterized and are highly abundant cell wall proteins found in the leaves. These are shown to inhibit *in-vitro* protein synthesis. Their toxicity to phytopathogenic fungi and the observation that their synthesis is induced by pathogenic infection suggest their involvement in plant defence.

(d) Pathogenesis Related Proteins

Plants evolved a variety of responses to protect themselves from pathogens. These are elicited as a reaction to pathogen attack (Hahlbrock & Grisebach, 1979). One of the extensively studied plant defence responses is the expression of pathogenesis related (PR) proteins (Carr & Klessig, 1989; Bowles, 1990; Bol et al., 1990). These are a group of host encoded inducible proteins whose synthesis is triggered by pathogen infection or other forms of stress. They were first discovered in tobacco mosaic virus (TMV) infection (Van Loon, 1983). In the past few years information has been accumulating about their biochemical properties, induction, regulation and possible role in plant disease resistance (Van Loon et al., 1987; Shinishi et al, 1987). During the induction of HR, a set of plant defence genes are activated in the host, and a large number of low molecular weight, soluble proteins are synthesized and released into the intercellular spaces. The presence of these proteins is correlated with induced resistance. Many of these same PR proteins have been detected in tobacco plants infected with pathogens other than TMV or treated with certain chemicals. In a number of other species also, new host encoded proteins similar to those of tobacco, have been detected after infection. Thus, the tobacco PR proteins can be considered as the prototypes of PR proteins in plants and the genes concerned appear to have been well conserved.

Tobacco PR proteins were classified into 5 families on the basis of electrophoretic and isoelectric point differences and are named PR1 to PR5. These are serologically related and share extensive homology of both nucleotide sequence of the genes and amino acid sequence of the proteins. Some of these proteins are acidic and the others basic.

Family PR1 comprises three acidic members (PR 1 a, PR 1 b, PR 1 c) which differ in isoelectric points. There are other serologically cross reacting PR 1-type proteins in other tobacco varieties. Inspite of extensive study, no biological function could be attributed to this class of proteins. cDNA clones and corresponding genomic clones for these genes have been isolated. It is found that these genes do not carry any introns but have interesting flanking sequences at their 5' region, which may play an important role in their expression.

Family PR2 proteins are known to be β-1, 3-glucanases (Kauffman et al., 1987). In the tobacco cultivars Samsun NN and Xanthinc there are three acidic members of the PR2 family called PR2, PRN and PRO (or Za, Zb and Zc). There is also a basic PR2 protein that resembles the basic, hormone-regulated β-1-3 glucanase produced by cultured tobacco cells.

Chitinases represent the PR3 family. These are specified by small gene families and manifest homology of the order of 65% in amino acid sequence despite differences in molecular weight and isoelectric points.

Biological function of PR 4 proteins is also not known. Although some members of this group are reported to have serological affinity with some PR2 proteins, the general consensus is that they represent a distinct class.

The PR5 family consists of the Thaumatin-like proteins. Thaumatin is the trivial name given to an intensely sweet tasting protein

found in the fruit of *Thaumatococcus danielli,* a tropical plant. Thaumatin shares a close homology with a bifunctional inhibitor from maize, which is active *in-vitro*, against trypsin and α-amylase. The bifunctional inhibitor of maize was implicated in defence against insects. PR5 proteins of tobacco share important structural features not only with thaumatin and maize bifunctional inhibitor but also with other proteins induced by stress.

PR proteins have been isolated from various other plants also. PR1 type of proteins are reported in powdery mildew infected barley and several PR proteins including chitinases are found in barley infected with powdery mildew as well as brome mosaic virus infected maize (White et al., 1987; Bryngelsson & Collinge, 1991). These proteins are observed only in HR and resemble those of tobacco. These are named infection related (IR) proteins and are found to be induced by a range of pathogens in barley (Scott et al., 1990).

In barley, the possible function of the proteins encoded by six IR mRNAs was investigated by sequencing the cDNAs. Two of the encoded proteins were investigated by sequencing the cDNAs. Two of the encoded proteins were identified as a PR1 protein and a β-1, 3 glucanase (Jutidamrongphan et al., 1991; Scott 1991).

In response to fungal, viral and viroid infection one PR1 like protein and several other acidic and basic proteins are induced in tomato. The PR1 like protein accumulated in tomato leaves infected with *Cladosporium fulvum* indicating a possible role for this in fungal resistance. Several other PR proteins also have been found in tomato plants manifesting resistance response to pathogen attack (Carr & Klessig, 1989).

Several PR proteins were found in the intercellular spaces of potato leaves (*Solanum tuberosum*) manifesting resistance to *Phytophthora infestans*. These include PR1 type (basic), PR2 type (acidic β-1, 3 glucanase) and the Thaumatin-like PR5 type. Induction of six different extracellular chitinases (PR3 type) is also observed in some varieties of potato (Kombrink et al., 1988).

In beans (*Phaseolus vulgaris*) showing HR to fungi and other pathogens, several PR proteins are induced. These could not be identified with any of the five classes of tobacco proteins. In cucumber (*Cucumis sativus*) cotyledons infected with viruses or *Colletotrichum lagenarium* several PR like proteins are synthesized during HR. One of these proteins accumulating in the intercellular spaces is an extracellular chitinase (PR3 type).

Hydrolytic enzymes : Among the PR proteins that have been studied, lytic enzymes chitinase and ß-1, 3-glucanase are speculated to play a crucial role in plant defence. As chitin, an insoluble, linear β-1, 4 linked polymer of N-acetylglucosamine and callose, a β-1, 3-glucan are common constituents of the cell walls of many phytopathogenic fungi. Plants do not contain chitin in their cell walls. However, they produce chitinase to protect themselves from chitin containing parasites (fungi and insects) (Bell, 1981; Boller, 1985). It is also reported that they occur in healthy plant cells (Hoj et al., 1989). These enzymes are known to be co-ordinately induced in several plants by ethylene, which is produced in response to infection stress in plants. The chitinases and β-1, 3-glucanases are proposed to be involved in plant defence through degrading fungal cell walls and releasing carbohydrate molecules with elicitor activity and thus triggering the synthesis of fungi toxic phytoalexins in the host plant.

From the structural analysis of plant chitinase genes, three classes of chitinases could be identified (Shinishi et al., 1987). Class I enzymes are basic isoforms with a

N-terminal cysteine-rich domain and a highly conserved catalytic domain. Class II enzymes lack the cysteine-rich domain but possess a catalytic domain which is homologous to that of class I enzymes. Class III enzymes stand apart, as they do not share homology with the other two classes. However, they are homologous to the acidic chitinases of cucumber and *Arbidopsis* (Samac et al., 1990).

In most plants, acidic and basic forms of chitinases are encoded by multi-gene families. These genes are differentially expressed during development and are induced by a variety of defence related and environmental stimuli. These enzymes also display distinct sub-cellular localization. The acidic isoforms are targeted extracellularly, while the basic isoforms acumulate in the central vacuole. This suggests that plants might have evolved distinct patterns of targeting chitinases to ward off the attack of invading fungi with a dual defence mechanism (Mauch & Staehelin, 1989).

Purified chitinases display strong antifungal activity against non-pathogenic fungi and a relatively weak anti-fungal activity against phytopathogenic fungi indicating that the *in-vivo* situation is more complex. In addition to chitinases, plants also synthesize chitin-binding lectins. Together, ß-1, 3-glucanases, chitinases and the binding lectins may form a defence system.

FUTURE POSSIBILITIES

From the foregoing account it is evident that plant disease resistance involves a resistance gene in the plant which responds specifically to the product of a single avirulence gene in the pathogen (Keen, 1990; De Wit, 1992). The avirulence genes encode elicitor molecules that bind to receptors located in the plasma membrane of the host plant cells. A gene of tomato that may code for a transmembrane receptor was recently isolated (Jones et al., 1994). There is evidence that transmembrane receptors are protein kinases (Braun & Walker, 1996; Xing et al., 1996). In the past few years a number of plant genes homologous to those encoding receptor protein kinases (RPKs) were identified and their products referred to as receptor-like protein kinases (RLKs) on the basis of their structural similarity to RPKs of animals. Plant RLKs characterized so far, autophosphorylate on serine and/or threonine residues. Their catalytic domains are very similar and distinct from those of other kinases. It is observed that several disease resistance genes encode proteins which are homologous to parts of RLKs (Braun & Walker, 1996). This indicates that RLKs may have a role in host defence response. For example, the *Cf-9* gene of tomato codes for a transmembrane protein with an extracellular domain of 28 leucine rich repeats (LRRs) and a short cytoplasmic tail (Jones et al., 1994). Proteins with LRRs and glycine rich protein kinases are known to be involved in signal transduction in animals (Kobe & Deisenhofer, 1994; Bossemeyer, 1994). It is possible that similar molecules are involved in the host-pathogen recognition. If the signal transduction pathway involved in the host-pathogen recognition is unraveled it will become possible to genetically engineerred plants carrying genes for pathogen incompatibility at the earliest event of infection, the recognition process. This becomes possible by involving a single signal component, the transmembrane receptor and is unlikely to interfere with the remaining biosynthetic mechanism. This system is apparently simpler than the two component sensor system suggested for evolving disease resistant plants (De Wit, 1992).

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