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The Possible Role of PR Proteins in Multigenic and Induced Systemic Resistance

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6.1 Introduction

There have been many studies dealing with PR proteins since their first discovery during analyses of the protein composition of tobacco mosaic virus (TMV)-induced hypersensitivity response in tobacco (Van Loon and Van Kammen, 1970) over 30 years ago. The amino acid composition of these proteins was quite variable, showing some of the proteins to be acidic and others to be basic, it was suggested that they may play an important role in the fate of pathogenesis (Kassanis et al., 1974; Van Loon, 1975). Fungi and bacteria were also discovered to induce similar new protein components in various plant species, particularly during incompatible combinations resulting in hypersensitive necrosis (Redolfi, 1983). Analyses of several of these showed a pattern of host responses in that they apparently consisted of one or more families of host-coded proteins, which were induced by different types of pathogens and abiotic stresses, and were most often of relatively low molecular weight, preferentially extracted at low pH, highly resistant to proteolytic degradation, and localized predominantly in the intercellular space of the leaf. These proteins coded for by the host plant but induced only in pathological or related situations have been termed pathogenesis-related (PR) proteins. While constitutively expressed proteins that show increases upon pathogen infection, such as oxidative and enzymes of aromatic biosynthesis are generally excluded, specific isoforms of such enzymes that are induced only as a result of infection have been grouped with PR proteins.

In this chapter, we will present evidences that timely accumulation of PR proteins during pathogenesis is a part of defense mechanisms in plants against pathogens and pests. Some of these proteins may have different roles in plant metabolism and/or may just be produced as a part of more generalized "housekeeping" regulatory systems during a plant–pathogen interaction. Specific isozymes of hydrolytic enzymes, on the other hand, demonstrate differential activity toward the substrate during the release of elicitor molecules from the pathogens. These isozymes may have evolved as a part of a suite of defense mechanisms in "naturally resistant"

plants, or plants considered to have higher basal resistance. Isozyme-based higher basal resistance may come about via the sensitization of plants to a particular pathogen, via the isozyme-mediated production of pathogen-derived nonspecific or specific elicitors that initiate the whole battery of defense mechanisms. It is important to recognize that plant defense mechanisms are complex and that more than one factor is involved in the successful existence of a plant species over the centuries in the face of abundant pathogen pressures. Pathogenesis can be considered the result of the failure of the plant's many and redundant defense-related mechanisms to activate in a timely manner to prevent or sharply contain pathogen infection.

6.2 Classification of PR Proteins

On the basis of their properties, the tobacco PR proteins were initially grouped into five families, and this classification is used in other plant species in which PR proteins are identified. The families are numbered and the different members within each family are assigned letters according to the order in which they are described. Thus the same designation for a PR protein in different plant species indicates that they belong to the same PR-family, but only reflects how many proteins of that family had been identified within those plant species. The genes encoding these proteins are designated as *ypr* followed by the suffix that corresponds to the protein. Because PR proteins are generally defined by their occurrence as protein bands on gels, gene and cDNA sequences cannot be fitted into the adopted nomenclature. Conversely, homologies at the nucleotide sequence level may be encountered without information on the expression or characteristics of the encoded protein. This leads to a complexity in comparative analysis of PR proteins from different species. Also, when new genes induced by pathogens or specific elicitors are identified they may be added to the existing families. Thionins (Bohlmann, 1994) and defensins (Broekaert et al., 1995), both families of small, basic, cysteine rich polypeptides were subsequently added to the families of PR proteins, based on this criterion. The identification of several such proteins with disparate properties necessitated the expansion of the classification, and addition of families. The nomenclature currently in use was proposed in 1994, and groups PR proteins into the 17 plant-wide families depicted in Table 6.1, on the basis of sequence homology and similarities in enzymatic and biological activities (Van Loon et al., 2002).

Localization of majority of the PR proteins in the intercellular spaces of leaves seems to guarantee contact with the invading pathogen before penetration. However, *in vitro* and *in vivo* analyses failed to show anti-pathogenic activity in any of the PR proteins associated with systemically induced resistance by a few *Pseudomonas* species (Van Loon, 1997), suggesting that they may not play a major role in defense in some systems (Pieterse et al., 1996). Accumulation of PR proteins to similar amounts in compatible as well as incompatible host interactions (Hoffland et al., 1995) suggests that PR proteins do not determine the resistance response in this particular host-pathogen interaction. However, constitutive accumulation of

TABLE 6.1. Recognized families of pathogenesis-related proteins.

Family	Type member	Properties	Reference
PR-1	Tobacco PR-1a	Antifungal	Antoniw et al. (1980)
PR-2	Tobacco PR-2	β-1,3-glucanase	Antoniw et al. (1980)
PR-3	Tobacco P, Q	chitinase type I,II, IV,V,VI,VII	Van Loon (1982)
PR-4	Tobacco 'R'	chitinase type I,II	Van Loon (1982)
PR-5	Tobacco S	thaumatin-like	Van Loon (1982)
PR-6	Tomato Inhibitor I	proteinase-inhibitor	Green and Ryan (1972)
PR-7	Tomato P69	Endoproteinase	Vera and Conejero (1988)
PR-8	Cucumber chitinase	chitinase type III	Métraux et al. (1988)
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase	Lagrimini et al. (1987)
PR-10	Parsley 'PR1'	'ribonuclease-like'	Somssich et al. (1986)
PR-11	Tobacco 'class V' chitinase	chitinase, type I	Melchers et al. (1994)
PR-12	Radish Rs-AFP3	Defensin	Terras et al. (1992)
PR-13	Arabidopsis THI2.1	Thionin	Epple et al. (1995)
PR-14	Barley LTP4	lipid-transfer protein	García-Olmedo et al. (1995)
PR-15	Barley OxOa (germin)	oxalate oxidase	Zhang et al. (1995)
PR-16	Barley OxOLP	oxalate oxidase-like	Wei et al. (1998)
PR-17	Tobacco PRp27	Unknown	Okushima et al. (2000)

low levels of hydrolytic enzymes in disease resistant varieties, as discussed below, may indicate a major role in defense against specific pathogens at least for this group of PR proteins (Lawrence et al., 2000).

Elucidation of the biochemical properties of the major pathogen-inducible PR proteins of tobacco and subsequent cloning of their genes revealed that many proteins with similarities to the classical PR proteins are present even in healthy plants (van Loon and van Strien, 1999). The term "PR-like proteins" was proposed to accommodate such protein homologues of PR proteins induced principally in a developmentally controlled, tissue specific manner. Sequence analyses and development of easily accessible database search tools in recent years have resulted in the identification of several proteins with sequence homology to established PR proteins and PR-like proteins (van Loon and van Strien, 1999). Though their inducibility and stress responses have not yet been established, they have been classified as PR and PR-like proteins based on their similarity. In contrast to the classical PR proteins, which are both intracellular and extracellular proteins, the PR-like proteins are mostly intracellular and localized to the vacuole (Linthorst et al., 1991), possessing enzymatic activities similar to the homologous PR proteins, but with different substrate specificities. The similarities in the activities of PR and PR-like proteins discovered in recent years makes it difficult to maintain their distinction. Some of these proteins have been shown to respond differently to different stimuli, and other proteins have shown organ specific regulation (Lotan et al., 1989; Memelink et al., 1990). The varied locations of the PR and PR-like proteins, and their differential induction by endogenous and exogenous signaling

compounds (Memelink et al., 1990) suggests that these proteins may have important functions extending beyond their apparently limited role in plant defense.

6.3 PR Proteins in Multigenic and Induced Systemic Resistance

Multigenic resistance, also known as "horizontal", "quantitative", or "polygenic" resistance, refers to plant disease resistance generated via interactions between the products of multiple plant genes, not a single R gene (Nelson, 1978; Simmonds, 1991). Multigenic resistance is considered to be nonspecific in that the plant and pathogen do not require matching R and *avr* genes for a timely plant defense response to occur. Multigenic-resistant plants which have been bred to resist a specific pathogen tend to resist a greater variety of pathogens and pathogen races than those bred or engineered to express particular R genes (Simmonds, 1991) and physical interaction of molecules present in the pathogens and their host receptors (Tang et al., 1996) lacks in this particular type of broad resistance.

Another category of disease resistance depends upon the induction of defenses following exposure to organisms or compounds. A variety of organisms, including virulent and avirulent pathogens (Tuzun et al., 1986, 1992, Tuzun and Kuć, 1991), mycorrhizal fungi (Borowicz, 1997) and nonpathogenic rhizobacteria (Tuzun and Kloepper, 1995; Benhamou et al., 1998) have all been observed to activate plant defense responses. Abiotic inducing agents include compounds isolated from plant pathogens (Wei and Beer, 1996; Norman et al., 1999) and a variety of chemicals (Fought and Kuć, 1996, Benhamou and Belanger, 1998). This general phenomenon is known as induced systemic resistance (ISR) (a term originally synonymous with systemic acquired resistance, or SAR) and generally results in a nonspecific resistance against a broad spectrum of pathogens and pests (Karban and Kuć, 1999). The extent of protection has sometimes been observed to vary (e.g., Manhandhar et al., 1999; Ton et al., 1999), and may depend upon the genotype and physiological condition of the plant, as well as the nature of the inducing agent used.

PR proteins may be induced in various tissues in response to a variety of stresses or stress-related plant hormones, including ethylene, osmotic stress, wounding, drought, high salt, and absicic acid (Horvath et al., 1998; Ponstein et al., 1994; Xu et al., 1994). The various conditions under which PR proteins occur are reminiscent of the conditions under which general stress response factors such as heat shock proteins are induced. However, PR proteins are not expressed or induced to any detectable levels in response to heat shock, suggesting that these proteins do not act as generic stress response factors. This suggests that the PR proteins may play roles that are more specific than those of general stress response factors.

In the remainder of this article, we intend to review evidence concerning the nature of multigenic and induced plant defense responses in terms of PR protein induction. The induction patterns and possible functions of specific genes, those encoding hydrolase isozymes in particular, related to these forms of resistance in several plant–pathogen systems will be discussed. A wide variety of enzymes have

been associated with disease resistance, only a few of which will be discussed in this article. For a more comprehensive review, see Van Loon and Van Strien (1999). For a review of PR proteins identified in cereals, see Muthukrishnan et al. (2001).

6.3.1 Hydrolytic Enzymes: Chitinases and β-1,3-glucanases

Families of PR proteins including hydrolytic enzymes include PR-2 (β-1,3-endoglucanases, Kauffman et al., 1987), PR-7 (endoproteinase, Vera and Conejoero, 1988) and the PR-3,-4,-8 and -11 families (chitinases, Legrand et al., 1987; Ponstein et al., 1994; Metraux et al., 1989; Melchers et al., 1994). The production of hydrolytic enzymes alone may not be sufficient for the protection of all plants from disease (e.g., Dalisay and Kuć, 1995 a,b). However, this does not mean that hydrolase isozymes are not involved in disease resistance, or that they do not play an important role in resistance to some pathogens. Hydrolytic enzymes may have a dual function in disease resistance: some isozymes will have direct antimicrobial effects against an invading pathogen. These isozymes, and/or others, may also accelerate and amplify the disease resistance process by generating hypersensitive response elicitors upon encountering a pathogen. Unfortunately, a great deal of the work regarding the role of specific enzymes in disease resistance fails to distinguish between the different isozymes that are present. Significant changes in the expression of a particular isozyme may go undetected.

Chitinases catalyze the hydrolysis of chitin, a linear polymer of β-1,4-linked N-acetylglucosamine residues that is the predominant constituent of fungal cell walls, nematode eggs, and mid gut layers of insects. Some plant chitinases also exhibit lysozymal activity (Boller, 1985; Dodson et al., 1993). Three classes of plant chitinases have been proposed based upon protein primary structure (Shinshi et al., 1990). The highly variable nature of chitinases, and the multiplicity of chitinase isozymes in plants, suggest that plant chitinase isozymes may carry out specific and differing roles. For example, chitinases, glucanases, and other PR proteins have been found to be induced as a consequence of cold stress and might be involved in resistance of winter wheat to snow mould infections (Gaudet et al., 2000). A *Lubinus albus* chitinase accumulates in response to salicylic acid, wounding, infection, and UV-C light (Regalado et al., 2000); and tobacco chitinases, glucanases and thaumatin-like proteins increased in response to UV-B light (Fujibe et al., 2004). Pre-treatment of tomato fruit with methyl jasmonate or methyl salicylate induces the synthesis of a variety of stress proteins, including chitinase and β-1,3-glucanase PR proteins, and subsequently increases the resistance of the fruit to chilling injury and infection by pathogens (Ding et al., 2002). Expression studies of various *Pinus* chitinase homologues showed the induction of multiple chitinase homologues after challenge by a necrotrophic pathogen (Davis et al., 2002), suggesting that different homologues may serve different functions in the plant. Some chitinase isozymes have antifungal activity while others do not, and the activity of antifungal chitinase isozymes isolated from tobacco (Sela-Buurlage et al., 1993) and tomato (Lawrence et al., 1996) has been found to be specific for certain pathogens.

Many plant pathogenic fungi contain β-1,3-glucans in their cell walls in addition to chitin. Chitinases and β-1,3-glucanases purified from tomato (Lawrence et al., 1996), tobacco (Sela-Buurlage et al., 1993), pea (Mauch et al., 1988) and the tropical forage plant *Stylosanthes guianensis* (Brown and Davis, 1992) have been found to have synergistic antifungal effects *in vitro*. The *in planta* antifungal effects of tomato and tobacco chitinases and β-1,3-glucanases have also been recorded (Benhamou et al., 1990; Benhamou, 1992), and chitinases and glucanases coexpressed in transgenic wheat were found to protect plants from infection by *Fusarium graminearum* under greenhouse conditions, although this resistance did not hold under field conditions (Anand et al., 2003). It has been suggested that the synergistic effects of these enzymes, and the specificity of their effects, may be attributed to the structure of a particular fungal cell wall. For example, the chitin layers of some fungal cell walls appear to be buried in β-glucans, rendering the chitin inaccessible to chitinases unless there is prior hydrolysis with β-1,3-glucanases (Benhamou et al., 1990).

Oligosaccharide elicitors of plant defense responses can be generated by chitinases and β-1,3-glucanases. Soybean β-1,3-glucanases (Keen and Yoshikawa, 1983; Ham et al., 1991) and specific isozymes of tomato chitinase and β-1,3-glucanase (Lawrence et al., 1996, 2000) have been demonstrated to generate elicitors from fungal pathogens. Tomato chitinases have also been shown to generate elicitors from germinating spores *of Alternaria solani*, but not the mature cell walls of this pathogen (Lawrence et al., 2000).

The tobacco PR-2 glucanase isozymes vary up to 250-fold in specific activity on various substrates, suggesting that their normal functions *in planta* may be quite diverse (Cote et al., 1991; Hennig et al., 1993). Interestingly, a β-1,3-glucanase found to accumulate in cultivars of resistant wheat could be involved in resistance to the Russian wheat aphid (Lintle et al., 2002). Glucanase and chitinase isozymes may also govern plant developmental processes not directly related to pathogenesis or stress resistance. For example, the expression studies of PR-2d in transgenic tobacco suggest that this protein functions developmentally in seed germination by weakening the endosperm (Vogeli-Lange et al., 1994). In yeast, a specific chitinase is secreted into the growth medium that is required for cell separation after division has taken place (Kuranda and Robbins, 1991), and has homology to a cucumber PR8 type III chitinase, suggesting that the yeast chitinase has functions in cell separation as well as defense. Specific chitinase homologues of PR3 and PR4 were found to be necessary for somatic embryogenesis to proceed beyond the globular stage (De Jong et al., 1992).

6.3.2 Antioxidant Enzymes

Plant cells are protected against damage from active oxygen species generated during the hypersensitive response by a complex antioxidant system, including enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase, and catalase (Zhang and Kirkham, 1994). Several species of active oxygen (O_2^-, H_2O_2, and OH^-) result from the reduction of molecular oxygen, and there are numerous

possible reactions which allow these species to interconvert (Elstner, 1987; Mader et al., 1980). Hydrogen peroxide, which has the longest half-life, provides a good estimate of the relative active oxygen level in the system. There is an opinion that elicitor- or pathogen-stimulated accumulation of H_2O_2 comes only from SOD-catalysed dismutation of superoxide radicals (Auh and Murphy, 1995). SOD and catalase are critical to the immediate level of H_2O_2 since they are involved in production and utilization of the molecule. The existence of multiple molecular forms of SOD, peroxidase, catalase, and other related enzymes and the variation in the activity of these during plant development suggests that each isozyme may have a separate role (Scandalios, 1993).

Specific peroxidase isoenzymes recognized as PR proteins in tobacco (Stintzi et al., 1993), that are identical to or homologous with a lignin-forming peroxidase, have been classified as PR-9. Peroxidases represent another component of an early response system in plants to pathogen attack (Mader and Fussi, 1982; Mader et al., 1980). The products of these enzymes, in the presence of a suitable hydrogen donor and hydrogen peroxide, can have direct antimicrobial and antiviral effects (Van Loon and Callow, 1983). The extracellular location of peroxidase isozymes stimulated during pathogen attack (Birecka et al., 1975), and their affinity for substrates involved in lignification, as well as the capacity of peroxidases to form hydrogen peroxide (Ride, 1975), suggest that peroxidase isozymes may also be involved in the formation of barrier substances which limit the extent of pathogen spread. Elicitation of peroxidase activity and lignin biosynthesis was observed in resistant pepper cell suspension cultures treated with the pathogen *Phytophthora capsici*, but not in susceptible cells (Egea et al., 2001). The release of superoxide and free radical intermediates during lignin polymerization (Grisebach, 1981) may be involved in restricting the growth of both fungal and bacterial pathogens (Klement, 1982; Ride, 1975; Tiburzy and Reisner, 1990). For example, antibacterial components active against *Xanthomonas oryzae* pv. *oryzae* were isolated from rice leaves and found to be lignin precursors (Reimers and Leach, 1991).

6.3.3 Thaumatins and Thaumatin-like Proteins (Osmotins)

The PR-5 family of proteins are referred to as thaumatins or thaumatin-like proteins due to their close or more varying sequence similarity to the intensely sweet protein thaumatin from *Thaumatococcus danielli* (Musthurkrishnan et al., 2001). PR-5 proteins have been shown to inhibit the growth of fungi *in vitro*, causing leakage of cytoplasmic material from ruptured hyphae (Vigers et al., 1991; Niderman et al., 1995). Two proteins highly induced by *Ascochyta rabiei* during infection of chickpea (*Cicer arietinum* L.) were identified as PR-5a and PR-5b proteins (Hanselle et al., 2001). An osmotin-like protein (OLP), which is a member of the thaumatin-like proteins, was purified from the seeds of *Benincasa hispida* (Shih et al., 2001). The homology of thaumatin-like PR-5 proteins with a bifunctional α-amylase/trypsin inhibitor from maize seeds (Richardson et al., 1987) suggests that these proteins could also play a role in protection against phytophagous insects.

Further characterization efforts to cloning and studying the expression of gene encoding osmotins resulted in demonstration that it is highly regulated by ABA and involved in adaptation to osmotic stress (Singh et al., 1987; 1989). Antifungal activity of osmotins appeared to be nonspecific against the cell wall from many fungi, although it is involved in permeabilization of plasma membrane to kill the cells (Abad et al., 1996). Although osmotins were antifungal to many strains of fungi, studies conducted using yeast strains with various resistance to this protein indicated that fungal cell wall proteins, encoded by *PIR* genes, are determinants of resistance to antifungal PR-5 proteins (Yun et al., 1997). Resistance to osmotin in yeast model system appeared to be strongly dependent on the natural polymorphism of the *SSD1* gene where it functions as post-transcriptional regulator of gene expression, cell wall biogenesis, and composition and deposition of PIR proteins (Ibeas et al., 2001). Deposition of such proteins as the fungal cell wall constituents that block the action of osmotin against *Aspergillus nidulans* requires the activity of G-protein mediated signaling pathway, and *A. nidulans* strains mutated to interfere this pathway also demonstrated increased tolerance to SDS, reduced cell wall porosity and increased chitin content in the cell wall (Coca et al., 2000). Further studies using yeast indicated that osmotins indeed have certain target molecules in the cell wall and several cell wall mannoproteins can bind to immobilized osmotin, suggesting that their polysaccharide constituent determines osmotin binding, demonstrating a causal relationship between cell surface phosphomannan and susceptibility of a yeast strain to osmotin (Ibeas et al., 2000). Overexpression of yeast glycoprotein in a plant pathogenic fungus *Fusarium oxysporum* f. sp. *nicotianae*, which is susceptible to osmotin, increased resistance to this antifungal protein and virulence in the fungal pathogen (Narasimhan et al., 2003), further indicating that osmotin plays a role in overall plant defenses against fungal pathogens.

6.3.4 Proteinase Inhibitors

The PR-6 proteins have been shown to be protease inhibitors (reviewed by Green and Ryan, 1972), and include wound-inducible proteinase inhibitors implicated in resistance to insect attack (Lawton et al., 1993). Proteinase inhibitor genes in *Nicotania glutinosa* that are induced in response to wounding as well as infection by TMV have been identified (Choi et al., 2000). Proteinase inhibitors have been shown to confer protection against a variety of insect and nematode pests when expressed in transgenic plants. For example, resistance to the cyst nematode *Globodera tabacum* (Urwin et al., 2002) and tobacco budworm (*Helothis virescens*, Pulliam et al., 2001) was conferred by proteinase inhibitors expressed in transgenic tobacco, and resistance to the potato cyst nematode *Globodera pallida* was conferred by a proteinase inhibitor expressed in transgenic potato (Urwin et al., 2001). Proteinase inhibitor proteins in plants may play roles other than protection against phytophagous insects and nematodes. Phloem-localized proteinase inhibitor proteins have been identified in *Solanum americanum*, which may be involved in regulating proteolysis in the phloem sieve elements (Xu et al., 2001).

Proteins induced under conditions of heat and drought stress have been found to have a putative proteinase inhibitor activity (Satoh et al., 2001), and it may be possible that PR-6 proteins have protective activity against abiotic stresses as well.

6.3.5 Ribonucleases

A recombinant white lupin *PR-10a* gene expressed in *Escherichia coli* exhibited a ribonucleolytic activity against several RNA preparations, including lupin root total RNA providing the first direct evidence of this enzymatic activity in a PR protein (Bantignies et al., 2000). Salicylate-inducible PR-10 genes from apple (*Apa*) were found to be also induced by wounding, ethephon, and exposure to virulent and avirulent fungi (Poupard et al., 2003). A PR-10 protein in western white pine that was associated with acclimation to cold was present in higher amounts in healthy pine needles than in infected ones, suggesting this protein may be involved in protecting frost-damaged plant tissues from pathogen attack (Yu et al., 2000). A similar protein in Douglas fir was found to increase during overwintering of plants but was not associated with acclimation to cold, and may accumulate in response to pathogen infection (Ekramoddoulah et al., 2000). A PR-10 protein was found to be induced in response to ozone and drought stress in birch, and its induction coincided with the formation of visible necrotic lesions and yellowing of leaves (Paakkonen et al., 1998). Ocatin, a member of the PR-10 family that is found in oca roots (*Oxalis tuberosa* Mol.) inhibits the growth of bacteria and fungi *in vitro*, and is expressed only in the pith and outer peel of the tuber, indicating a role in protecting tubers from pathogen attack (Flores et al., 2002). PR-10 genes that accumulate after pathogen attack have also been found in rice (McGee et al., 2001), sorghum (Lo et al., 1999), and alfalfa (Borsics and Lados, 2002). High sequence similarity between ribonuclease from ginseng callus cultures and fungus-elicited PR-proteins in parsley further indicates that at least some of the intracellular PR-proteins are ribonucleases (Moiseyev et al., 1994).

6.3.6 Thionins

The PR-13 family consists of thionins, small (5000 Da) sulfur-rich plant proteins that exert toxicity in various biological systems by destroying membranes (Bohlmann, 1994). They are synthesized as preproteins and secreted into vacuoles, protein bodies, and the plant cell wall, and may be subsequently released upon pathogen infection, and display antifungal and antibacterial activity *in vitro* (Bohlmann, 1994; Terras et al., 1995) The expression of thionins in transgenic plants has been found to protect against pathogenic bacteria in rice (Iwai et al., 2002) and *A. thaliana* (Epple et al., 1997), and thionin concentrations in cell walls have been found to be higher in disease-resistant cultivars of barley and wheat (Ebrahim-Nesbat et al., 1994). *Arabidopsis* mutants constitutively expressing the thionin (*cet*) gene *Thi2.1* showed spontaneous formation of necrotic lesions and

an upregulation in the PR-1 gene, reactions that are associated with a salicylate-dependent induced systemic resistance response (Nibbe et al., 2002). Nonspecific resistance to snow moulds and other fungi has been likened to the expression of γ-thionin in winter wheat (Gaudet et al., 2003).

6.4 Patterns of Expression of Chitinases, Glucanases and Peroxidases in Multigenic Resistant and Induced Resistant Plants

In this section, the manner in which hydrolytic and antioxidant enzymes are expressed in plants, which express multigenic resistance and plants in which systemic resistance has been induced, will be compared. Three plant systems (tobacco, tomato, and cabbage) will be discussed in some detail, while work in other plant systems will be mentioned briefly at the end.

6.4.1 Tobacco

Resistance to *Peronospora tabacina* (blue mold) in tobacco is considered to be due to a few genes acting in an additive fashion (Rufty, 1989). Several breeding lines, which have been developed by the use of intraspecific hybridization of wild *Nicotania* species *to N. tabacum* by Rufty (1989) were used for studying the role of preformed hydrolytic enzymes in tobacco. Results from SDS-PAGE and Western blot analyses consistently revealed the presence of chitinase and β-1,3-glucanase isozymes prior to pathogen attack, as well as an earlier induction of isozyme accumulation following attack, in the resistant lines (Tuzun et al., 1997). Enzyme activity assays closely correlated with the Western blot analysis (Robertson, 1995).

Induced systemic resistance to *Peronospora tabacina* (blue mold) occurs naturally under field conditions (i.e., in plants not inoculated by human beings) (Tuzun et al., 1992). Inoculation of tobacco with *Peronospora tabacina* spores or tobacco mosaic virus (TMV) resulted in the induction of systemic resistance against a variety of pathogens (McIntyre et al., 1981) and the accumulation of β-1,3-glucanase and chitinase isozymes prior to foliar inoculation (Tuzun et al., 1989; Ye et al., 1990; Pan et al., 1991,1992). Similar results were observed for tobacco inoculated with viruses, PGPR, or various chemical inducers (Maurhofer et al., 1994, Schneider and Ullrich, 1994; Lusso and Kuć, 1995). Increases in lysozyme, peroxidase, polyphenol oxidase, and phenylalanine ammonium lyase activity, correlated with the induction of ISR, have also been reported (Ye et al., 1990; Schneider and Ullrich, 1994). Inhibition of fungal pathogen growth was found to precede host cell necrosis in induced tobacco, and it is thought that this might be due to the production of defense response elicitors by hydrolytic enzymes (Ye et al., 1992).

Elevated constitutive expression of an endochitinase gene from *Trichoderma viride* in tobacco and potato resulted in significant protection against multiple

fungal pathogens (Lorito et al., 1998). Reduced levels of anionic peroxidase, however, did not result in reduced lignification in transgenic tobacco (Lagrimini et al., 1997).

6.4.2 Tomato

Tomato breeding lines and several plant introductions of *Lycopersicon* spp. have already been identified in several studies with heritable foliar resistance to the early blight pathogen *Alternaria solani*, conferred by the presence of multiple genes (Barksdale and Stoner, 1973; Gardner, 1988; Maiero et al., 1990; Maiero and Ng, 1989; Nash and Gardner, 1988). These studies also suggest that expression of a resistant phenotype in a given individual relies on various genetic interactions of an additive and/or epistatic nature. All tomato breeding lines resistant to *A. solani* were found to express significantly higher consitutive levels of chitinase and β-1,3-glucanase isozymes than susceptible plants (Lawrence et al., 1996, 2000). The same 30 kDa chitinase isozyme expressed to a high level in resistant lines was also found to accumulate more rapidly, and to significantly higher levels, in the resistant lines than in the susceptible ones during pathogenesis (Lawrence et al., 1996). The resistant tomato lines expressing elevated levels of chitinase and β-1,3-glucanase isozymes are also able to produce a greater number of, or more effective, elicitors of the hypersensitive response from *A. solani* cell walls than susceptible tomato lines (Lawrence et al., 2000). It is thought that the higher constitutive expression of hydrolytic enzymes might therefore contribute to disease resistance to *A. solani* via the more rapid and greater production of oligosaccharide elicitors upon contact with the pathogen, that in turn activate other defense mechanisms. More rapid accumulation of chitinases in resistant plants during incompatible tomato–pathogen interactions have also been observed *in planta* by other researchers (Benhamou et al., 1990). Two genes encoding basic chitinases, which accumulate during pathogenesis in tomato have been sequenced, and the promoter region of one of these genes cloned (Baykal and Tuzun, unpublished data). The manner in which the gene is regulated is currently being determined.

Tomato plants immunized with β-amino butyric acid (BABA) accumulated β-1,3-glucanase and chitinase (Cohen et al., 1994), while tomato plants immunized with 4-hydroxybenzoic hydrazide, saliclylic hydrazide, or 2-furoic acid accumulated an acidic peroxidase (Miyazawa et al., 1998). Interestingly, this peroxidase was not produced as a result of pathogenesis or wounding, suggesting that different kinds of inducing agents may have different effects on plant physiology. Enkerli et al. (1993) reported correlations between increased tomato chitinase activity, but not β-1,3-glucanase activity, with induction of resistance. Similarly, correlations between induced resistance in tomato and increased production of various antifungal proteins or activity of peroxidases, but not β-1,3-glucanases, have been reported (Anfoka and Buchenauer, 1997). Treatment of tomato roots with the mycoparasite *Pythium oligandrum* (Benhamou et al., 1997), chitosan and *Bacillus pumilis* (Benhamou et al., 1998) or with benzothiadiazole (Behnamou and Belanger, 1998)

was able to trigger and amplify plant defense responses to infection with a fungal pathogen, including the deposition of newly formed barriers containing callose and phenolic compounds.

6.4.3 Cabbage

A high level of resistance to the black rot pathogen, *Xanthomonas campestris* pv. *campestris* (XCC), was observed decades ago in the cabbage cultivars Early Fuji and Hugenot (Bain, 1952), and the heritable nature of this resistance was found to involve one major and several modifying genes (Bain, 1955). Cabbage varieties demonstrated to be resistant to a virulent strain of XCC have been found to constitutively express higher levels of the chitinase-lysozyme isozyme CH2 than susceptible cabbage varieties (Dodson et al., 1993). The level of CH2 expression was correlated with the extent of black rot disease resistance. Acidic protein extraction and denaturing electrophoresis identified at least 12 acid-extractable proteins which accumulated in both black-rot resistant and susceptible varieties following XCC infection; however, accumulation was early and more pronounced in the resistant varieties (Tuzun et al., 1997). The chitinase-lysozyme CH2, as well as peroxidase and superoxide dismutase isozymes, accumulate more rapidly and to a greater extent following inoculation with XCC than susceptible varieties (Dodson et al., 1993; Gay and Tuzun, 2000b). Increases in chitinase, lysozyme, peroxidase, and superoxide dismutase activities have also been correlated with increased expression of these isozymes. Higher peroxidase activity in the hydathodal fluids of black rot-resistant cabbage varieties than in susceptible ones was related to increased suppression of XCC growth in the hydathodal fluids (Gay and Tuzun, 2000a). Localized accumulations of peroxidase may function to protect plants against XCC infection, since this pathogen initially invades cabbage via the hydathodes (Staub and Williams, 1972).

Incompatible interactions with *X. campestris* pv. *vesicatoria* and a less pathogenic strain of XCC were sufficient to induce systemic resistance in cabbage against pathogenic isolates of XCC under both greenhouse and field conditions (Jetiyanon, 1994). Immunized plants produced chitinase/lysozyme, β-1,3-glucanase, osmotin, and other pathogenesis-related proteins earlier and in greater quantities than did nonimmunized plants (Tuzun et al., 1997).

6.4.4 Other Systems

Higher constitutive expression of chitinases and/or glucanases in disease-resistant plants relative to susceptible ones has also been noted in barley (Ignatius et al., 1994), grape (Busam et al., 1997), and potato (Wegener et al., 1996). Increases in the expression or activity of chitinase and/or β-1,3-glucanase isozymes in disease resistant plants after pathogen challenge have been reported in barley (Ignatius et al., 1994), pea (Vad et al., 1991), and wheat (Liao et al., 1994; Siefert et al., 1996, Kemp et al., 1999). Chitinase expression increased in wilt-resistant cotton plants following infection by *Verticillium dahliae*, but β-1,3-glucanase expression

did not (Cui et al., 2000). Two wheat genes that encode proteins PR-1.1 and PR-1.2, expression of which was induced upon infection with either compatible or incompatible isolates of the fungal pathogen *Erysiphe graminis*, were identified (Molina et al., 1999). Two new PR-4 family proteins (named wheat win3 and wheat win4) showing distinct antifungal activity were identified from wheat (Caruso et al., 2001). A similar protein has been identified from bean leaves, with similarity to PR1 like protein and glucanase, and a thaumatin like protein (Del Campillo and Lewis, 1992). Thionins, defensins, PR-like chitinases, thaumatin like proteins isolated from wheat, barley, sorghum, oats, and maize have antifungal activity (Hejgaard et al., 1991; Vigers et al., 1991).

Increases in the expression and activity of chitinases, β-1,3-glucanases and/or peroxidases after the induction of ISR has also been reported in cotton (Dubery and Slater, 1996), wheat (Liao et al., 1994; Siefert et al., 1996), rice (Manandhar et al., 1999), coffee (Guzzo and Martins, 1996), grape (Busam et al., 1997), cucumber (Schneider and Ullrich, 1994; Ju and Kuć, 1995; Dalisay and Kuć, 1995a,b), bean (Dann et al., 1996; Xue et al., 1998), pepper (Hwang et al., 1997), chestnut (Schafleitner and Wilhelm, 1997), *Cotoneaster watereri* (Mosch and Zeller, 1996), and *Stylosanthes guianensis* (Brown and Davis, 1992). Kogel et al. (1994) reported that ISR in barley is associated with increases in PR-1, peroxidase and chitinase proteins, but not β-1,3-glucanase. Although it appears to be a rather specific case, ISR induced in radish by *Pseudomonas fluorescens* has yet to be explained since no pathogenesis-related proteins accumulate and no changes in cell wall composition occur (Steijl et al., 1999).

Chitosanases, chitinases and β-1,3-glucanases were observed to accumulate in infected spruce seedlings (Sharma et al., 1993), and in the vicinity of the pathogenic fungus in infected spruce and pine (Asiegbu et al., 1999). These observations indicate that the defense responses of gymnosperms are similar to those of angiosperms. Induced resistance to pathogenic fungi in mature Norway spruce trees was found to be localized to the immunized bough rather than being systemic throughout the plant, but this was attributed to the size of the plant rather than a fundamental difference in induced resistance mechanisms (Krokene et al., 1999).

6.5 Regulation in PR Gene Expression

How pathogen infection leads to PR gene expression is as yet not well understood. Some of this is due to the fact that PR gene expression appears to be induced by environmental stimuli (e.g., cold stress, ultraviolet light) as well as developmental cues. Interestingly, the systemic induction of BiP, a lumenal binding protein in tobacco that is required for the normal induction of PR gene expression, occurs prior to the induction of PR genes (Jelitto-van Dooren et al., 1999)

It is possible that some PR proteins, specifically hydrolytic enzymes, act to stimulate an appropriately rapid or intense defense response by amplifying the concentration of nonspecific elicitors that go on to stimulate defense responses, including the production of more hydrolytic PR proteins. A role for hydrolytic

PR proteins as defense response signal amplifiers would be consistent with their generally rapid induction in response to stressful pathogenic infections.

6.5.1 Elicitors of PR Gene Expression

A variety of microbially-produced surface or secreted molecules have been identified as elicitors of PR gene induction, including oligosaccharides, oligochitin and oligoglucan fragments, extracellular glycoproteins and peptides, lipopolysaccharide from *Burkholderia cepacia*, and Avr proteins derived from bacterial and fungal pathogens (Boller and Felix 1996; Coventry and Dubery, 2001).

Avr proteins are specific elicitors, meaning that a pathogen expressing that product is recognized by a host plant expressing the corresponding resistance gene (*R*), which then activates the disease resistance mechanisms of the host (Staskawicz et al., 1995; Bent, 1996). Activation of tomato PR genes by the *Avr9* gene product of *Cladosporium fulvum* (Wubben et al., 1994), and activation of barley PR gene expression by *Rhychosporium* NIP1 Avr protein (Rohe et al., 1995) provide examples of defense responses induced by specific elicitors. Nonspecific elicitors (i.e., elicitors other than Avr proteins) also seem to be detected by receptors, which then stimulate a defense response. The receptor of a soybean β-glucan elicitor (GE) that induced phytoalexin biosynthesis was identified (Umemoto et al., 1997), and a parsley glycoprotein secreted by *Phytophthora sojae* has been shown to elicit ion channel openings and expression of defense genes including PR genes (Nurnberger et al., 1994; Ligterink et al., 1997).

6.5.2 Activation of PR Gene Expression

Secondary signal molecules such as reactive oxygen species, salicylic acid, ethylene, and jasmonates have been shown to induce PR gene expression (Delledone et al., 1998; Durner et al., 1998; Ecker, 1995). However, it is uncertain whether this induction requires secondary messengers. Proteinase inhibitor (Pin) of tomato is inhibited by ethylene and jasmonates, whereas these secondary signal molecules enhance tobacco osmotin, PR1, and tomato Pin2 (O'Donnell et al., 1996; Farmer et al., 1994). Using an inducible gene expression system, McNellis et al. (1998) directly expressed the AvrRpt2 protein in *Arabidopsis* and stimulated a hypersensitive response as well as induction of PR-1 gene expression.

A DNA microarray analysis of gene-expression changes in *Arabidopsis thaliana*, under 14 different ISR-inducing or ISR-repressing conditions, was used to derive groups of genes with common regulation patterns (regulons). A common promoter element in genes of the PR-1 regulon that binds members of a plant-specific transcription factor family was identified (Maleck et al., 2000). The promoter regions of two peach β-1,3-glucanase genes, designated *PpGns1* and *PpGns2*, identified to be highly expressed upon exposure to *Xanthomonas campestris* pv. pruni, contain elements similar to the *cis*-regulatory elements present in different stress-induced plant genes (Thimmapuram et al., 2001). Receptor-mediated recognition of *Phytophthora sojae* may be achieved

through a 13 amino acid peptide sequence (Pep-13) present within an abundant cell wall transglutaminase, which initiates a defense response that includes the transcriptional activation of genes encoding pathogenesis-related (PR) proteins (Kroj et al., 2003). Identification of *cis* regulatory elements mediating pathogen-induced PR gene expression suggested that the regulation primarily occurs at the level of transcription. The *cis* regulatory elements include GCC box (AGCCGCC), W box (TTGACC or TGAC-[N]x-GTCA), MRE-like sequence (A[A/C]C[A/T]A[A/C]C), G box (CACGTG), SA responsive element (SARE, TTCGACCTCC), and a parsley 11bp element mediating PR2 gene expression. Of these only the GCC box and the W box are extensively studied.

GCC box initially denoted as an ethylene responsive element has been identified in the promoter of a number of basic PR genes (Hart et al., 1993). The absence of GCC box in other ethylene responsive genes suggests that GCC box may be associated with the defense response mediated by ethylene. The GCC box confers ethylene-induced transcription of tobacco *gln2* and *PRB-1b* genes. Also, a 140 bp fragment that contains the GCC box from osmotin promoter is necessary to confer responsiveness of osmotin to various stimuli (Ragothama et al., 1997). Thus, GCC box might be a point of cross talk between various signal transduction pathways.

The promoter of an *Arabidopsis* basic PR1-like gene, AtPRB1, establishes organ-specific expression pattern and responsiveness to ethylene and methyl jasmonate (Santamaria et al., 2001). Identification of GCC box binding proteins (EREBP1-4) containing a conserved domain responsible for binding to the GCC box suggests that ethylene further induces the expression of the EREBP genes. Homologues of the EREBP genes have been identified from several species (Kitajama et al., 2000). An *Arabidopsis* EREBP homolog (AtERF1), which acts downstream of EIN3 (a component of the ethylene signaling pathway), has been identified to activate PR gene expression (Chao et al., 1997; Solano et al., 1998). EIN3 has subsequently been shown to be a transcriptional regulator of AtERF1.

A *Glycine max* gene encoding the ethylene-responsive element-binding protein 1 (GmEREBP1) has been shown to have differential expression during soybean cyst nematode infection (Mazarei et al., 2002). Three tomato Pto interacting proteins (Pti), with homology to the tobacco EREBPs have been identified, and shown to bind the GCC box of the tobacco *gln2* gene (Zhou et al., 1997). This suggests that Pti4/5/6 and EREBPs act in the R gene pathway. Ptis have also been shown to be highly regulated proteins. Pto kinase interacts directly with Pti4/5/6, and phosphorylates Pti4 protein specifically, to enhance the ability of Pti4 to activate expression of GCC-box PR genes in tomato (Gu et al., 2000). While *Pti4* is constitutively expressed and shows increased accumulation on infection, *Pti5* transcript is induced only upon infection. Pti4 also responds to mechanical and osmotic stress. A protein kinase that regulates the expression of PR genes has also been identified from rice. The rice mitogen-activated protein kinase (OsMAPK5) has been shown to negatively modulate PR gene expression (PR1 and PR10) and broad-spectrum disease resistance (Xiong and Yang, 2003). Analysis of the *Arabidopsis* PDF1.2 promoter shows a GCC box, and that the promoter confers pathogen and jasmonate

responsiveness. This demonstrates that AtPDF1.2 gene is a target for EREBP/Pti class of transcription factors (Wu et al., 2002). Expression of *Pti4*, *Pti5*, or *Pti6* in *Arabidopsis* activated the expression of the salicylic acid-regulated genes *PR1* and *PR2* (Gu et al., 2002).

The W box has been identified in parsley *PR1* and *PR2*, tobacco chitinase, asparagus *PR1*, potato *PR10a*, and maize *Prms*. Promoter deletion analysis showed that the W box is required for the elicitor-induced response of these PR genes. The W box has also been identified in other pathogen responsive genes suggesting a wider role for this *cis* element (Rushton and Somssich, 1998; Somssich, 2003). Rushton et al. (1996) identified a family of parsley proteins, which bind the W box, activating the expression of genes containing the W box. The *Arabidopsis* WRKY protein ZAP1 can activate a W box indicating that ZAP1 is capable of *trans*-activating W box containing genes (De Pater et al., 1996). The parsley *WRKY1* gene has been shown to bind the W box elements and act as a transcriptional activator (Eulgem et al., 1999).

Efforts are on to analyze the regulation of gene expression mediated by other regulatory elements identified to modulate PR gene expression. In *Arabidopsis*, NPR1 was originally discovered as a key regulatory protein that functions downstream of SA in the ISR. Upon induction of ISR, NPR1 activates PR-1 gene expression by physically interacting with a subclass of basic leucine zipper protein transcription factors (TGA/OBF family of transcription factors) that bind to promoter sequences required for SA-inducible PR gene expression (Chao et al., 1997; Zhou et al., 2000; Van Wees et al., 2000). In addition, analysis of the *Arabidopsis* mutant *npr1*, which is impaired in SA signal transduction, revealed that the antagonistic effect of SA on JA signaling requires the NPR1. Nuclear localization of NPR1 indicating that cross-talk between SA and JA is modulated through a novel function of NPR1 in cytosol (Spoel et al., 2003). A negative regulator of ISR, sni1 (suppressor of *npr1*) has been identified in a suppressor screen of *npr1* mutant (Li et al., 1999). Epistatic analysis has identified *cpr5* and *cpr1* as genes acting upstream of SA production and the *npr1* and *cpr6* downstream of SA production (Clarke et al., 1998; Dong, 1998). It also shows that *cpr5* is a negative regulator of the hypersensitive response, and *cpr1* is a negative regulator of SA biosynthesis. In addition the classical PR genes, defensin (PDF1.2) gene and thionin (Thi2.1) are constitutively expressed in *cpr5* and *cpr6* mutants. In contrast, the *cpr1* mutant accumulates only the classical PR genes and not the PDF1.2. A double mutant (npr1: cpr5) accumulated the PDF and not the PR genes, suggesting that expression of PDF is independent of NPR1. Thus, the activation of *Arabidopsis* defense genes appears to follow two separate pathways: an NPR1 dependent pathway for PR1, PR2, and PR5, and an NPR1 independent pathway for PDF and Thi2. Genetic analysis also suggests that CPR6 may be responsible for the crosstalk between SA mediated signaling pathway and the jasmonates/ethylene mediated signaling pathway (Clarke et al., 1998). It appears that overexpression of regulatory genes for induced systemic resistance that results in broad spectrum of resistance (Cao and Dong, 1998) also involves accumulation of PR proteins. A protein identified as the silencing element binding factor (SEBF) that binds elements in the promoter region of potato

PR10a was shown to act as a transcriptional repressor of PR10a expression (Boyle and Brisson, 2001). Elicitor-induced activation of the potato PR-10a requires the binding of the nuclear factor PBF-2 (PR-10a binding factor 2) to an ERE (elicitor response element) in the promoter region, and thus acts as a transcriptional regulator (Desveaux et al., 2000).

6.6 Conclusion

Timely accumulation of PR proteins during pathogenesis can be suggested as a part of defense mechanisms in plants against pathogens and pests. Some of these proteins may have a different role in plant metabolism and/or may just occur there as a part of regulatory systems overall happening during the plant–pathogen interactions. Specific isozymes of the hydrolytic enzymes, on the other hand, which demonstrate differential activity toward the substrate during the release of elicitor molecules from the pathogens may have been evolved as a part of defense mechanisms in "naturally resistant plants". Such isozymes may be bred into the resistant lines of crop varieties act as recognition mechanism to initiate the whole battery of defense mechanisms. It is also clear that some of PR-proteins such as osmotins and hydrolytic enzymes have a direct involvement in reduction of pathogenesis as evidenced by genetic studies as well as microscopic observations. However, it is important to recognize that plant defense mechanisms are complex and more than one factor is involved in the successful existence of plant species over the centuries under the abundance of numerous organisms that can be potentially harmful to plants. Nevertheless pathogenesis is an exception, and is a result of failure of many pathways to be activated in a timely manner. PR proteins are certainly there for a reason, whether they are a part of a major defense mechanisms or not, according to the inducer, they are a part of induced systemic resistance and more studies will further show that they may be the reason of successful breeding efforts, which we have been doing over the centuries to breed disease resistant varieties carrying more than one gene for resistance.

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