LITERATURE REVIEW

Plants are compelled to withstand stresses of all kinds, be it biotic, abiotic or anthropogenic as a consequence of their immobility. Higher plants protect themselves from various stresses, such as pathogen attack, wounding, application of chemicals including phyto-hormones and heavy metals, air pollutants like ozone, ultraviolet rays, and harsh growing conditions. Such constraints lead to production of a wide array of defense compounds, which are either induced or preformed. One of the ways in which plants respond to biotic and/or abiotic stress factors are the accumulation of various novel proteins collectively referred to as pathogenesis-related proteins or PR-proteins. Such protective plant proteins specifically induced in pathological or related situations have been intensively studied from the agricultural interest. On the other hand, many of the reserve proteins accumulated in seeds and fruits take the constitutive defense function against microbial pathogens and invertebrate pests in addition to their storage function. These inducible or constitutive defense mechanisms of higher plants are relatively conserved in the course of evolution. Accordingly, most plants produce or accumulate similar proteins under certain situations irrespective of their morphological differences. These proteins were first observed in tobacco, *Nicotiana tabacum*, cultivars reacting hypersensitively to tobacco mosaic virus (Van Loon and Van Kammen, 1970; Gianinazzi *et al.*, 1970). The occurrence of these proteins was not pathogen specific but determined by the type of reaction of the host plant, indicating that these proteins were of host origin. Since the proteins were induced under specific pathological conditions, they were named 'pathogenesis-related proteins' abbreviated as PRs. PRs were found in small amounts in senescing leaves of flowering plants and in relatively larger quantities when necrosis was more severe. This led to the assumption that these polypeptides were stable proteolytic breakdown products of larger leaf proteins.

By definition, these proteins abbreviated as PRs, were described as "proteins coded for by the host plant but induced specifically only in pathological or related situations" (Van Loon and Van Kammen, 1970). PRs are most often of low molecular weight, selectively extractable in low pH, highly resistant to proteolytic degradation/ or endogenous proteases and localized predominantly in the intercellular space (Kassanis *et al.*, 1974). PRs have been found in several plant species belonging to various families (Table 1).

Table 1: Plant species in which pathogenesis-related proteins have been identified

Family	Plant species
Amaranthaceae	*Gomphrena globosa*
Chenopodiaceae	*Chenopodium amaranticolor, C. quinoa, Beta vulgaris*
Compositae	*Gynura aurantiaca, Helianthus annuum*
Cruciferae	*Arabidopsis thaliana, Brassica nigra, B. juncea, B. napus, B. rapa, Raphanus sativus*
Cucurbitaceae	*Cucumis sativus, C. melo, Cucurbita maxima, C. pepo*
Gramineae	*Hordeum vulgare, Zea mays, Avena sativa, Oryza sativa, Triticum aestivum*
Malvaceae	*Gossypium hirsutum*
Papilionaceae	*Medicago sativa, Phaseolus vulgaris, P. lunatus, Cicer arietinum, Vigna unguiculata, V. radiata, Arachis hypogea, Lablab purpureus, Pisum sativum, Glycine max*
Rutaceae	*Citrus sinensis*
Solanaceae	*Capsicum annuum, Petunia, Solanum demissum, S. nigrum, S. tuberosum, S. dulcamara, Nicotiana debneyi, N. glutinosa, N. rustica, N. sylvestris, N. langsdorfii, N. plumbaginifolia, N. tomentosiformis, N. tabacum, Lycopersicon esculentum*
Umbelliferae	*Petroselinum crispum, Apium graveolens*
Vitaceae	*Vitis vinifera*
Pinaceae	*Picea abies*

The introduction of polyacrylamide electrophoresis by Davis (1964) and Ornstein (1964) by which proteins could be separated on the basis of their combination and charge was a chief innovation in unearthing PRs – the new protein components induced by TMV in hypersensitively reacting tobacco. The protein extracts from *N. tabacum* revealed thirty bands and additional separation was achieved with varying concentrations of the acrylamide in the gels and the electrophoretic pattern was represented by densitometric tracings (Van Loon and Van Kammen, 1970). Moreover, it was observed that the same proteins were induced in tobacco by different viruses, whereas different proteins appeared upon infection in *N. glutinosa*. Aided by selective extraction methods (Van Loon, 1976; Gianinazzi *et al*, 1977; Parent and Asselin, 1984) it was established that not only viruses but also

fungi and bacteria were able to induce similar protein components in various plant species particularly in incompatible combinations resulting in hypersensitive necrosis. Proteins that are induced exclusively during disease development in compatible host-pathogen combinations have hardly been considered as PRs. PRs have been grouped into families on the basis of similarities in molecular weights, amino acid composition and serological properties confirmed by nucleotide sequencing of corresponding cDNAs (Van Loon, 1975).

Nomenclature

The classification has set a convenient standard for other plant species, in which these family numbers now similarly designate PRs with properties homologous to tobacco PRs. Initially the PRs were grouped in to five main classes consisting of the 10 major acidic PRs of tobacco characterized both by biochemical and molecular biological techniques and designated as PR-1 to -5 (Gianinazzi *et al.*, 1977; Van Loon 1985).

A unifying nomenclature was proposed based on their grouping into eleven families, classified for tobacco and tomato, sharing amino acid sequences, serological relationships and/ or enzymatic or biological activity. The criteria for inclusion of new families of PRs were (i) protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and (ii) induced expression must have shown to occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combination must have been confirmed independently in different laboratories (Van Loon and Van Strien, 1999). Each PR family is numbered and the individual family members are assigned lower case letters in the order in which they are described. In accordance with the recommendations of the Commission for Plant Gene Nomenclature, PR-genes are designated as *ypr*, followed by the same suffix as of the family. Later on three more peptides, which were capable of inducing defense responses of plants, were identified. These three families (PR-12, -13 and -14) comprise the pathogen induced plant defensins (PR-12) (Fraser, 1981) thionins (PR-13) (Antoniw *et al.*, 1981) and lipid transfer proteins or LTPs (PR-14) (Garcia-Olmedo *et al.*, 1995). So far, 17 families of PRs have been recognized (Table 2). However, the properties of all these proteins have not yet been elucidated.

Table 2. Recognized families of pathogenesis-related proteins

Families	Type member	Properties	Gene symbol
PR-1	Tobacco PR-1a	antifungal	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* ('*Glb*')]
PR-3	Tobacco P, Q	chitinase type I,II, IV,V,VI,VII	*Ypr3, Chia*
PR-4	Tobacco 'R'	chitinase type I, II	*Ypr4, Chid*
PR-5	Tobacco S	thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	proteinase-inhibitor	*Ypr6, Pis (Pin')*
PR-7	Tomato P_{69}	endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	chitinase type III	*Ypr8, Chib*
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase	*Ypr9, Prx*
PR-10	Parsley 'PR1'	'ribonuclease-like'	*Ypr10*
PR-11	Tobacco 'class V' chitinase	chitinase, type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	thionin	*Ypr13, Thi*
PR-14	Barley LTP4	lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxOa (germin)	oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	'oxalate oxidase-like'	*Yrp16*
PR-17	Tobacco PR27	unknown	*Yrp17*

Besides proteins newly defined mRNAs (cDNAs) are often considered as additional members of the existing families where shown to be induced by pathogens or specific elicitors. However, because PRs are generally defined by their occurrence as protein bands on gels, and classified within each family once the protein has been characterized, cDNA or genomic sequences without information on the corresponding protein cannot be fitted in to the adopted nomenclature. Thus for naming it is necessary to gather information at both the nucleic acid and the protein level when dealing with a stress-related sequence falling within the definition of PRs. Conversely, homologies at the cDNA or genomic level may be encountered without information on the expression or characteristics of the encoded protein. Such sequences obviously belong to the PR-type families, but yet cannot, be considered to correspond to pathogen-induced PRs and named accordingly. In more than a few situations, it is difficult to distinguish PRs from related proteins/ mRNAs that are

present in some organs or appear during specific developmental stages. Homologous proteins/ mRNAs in healthy tissues in which no induction by pathogen infection has yet been demonstrated, are to be termed PR-like proteins (PRLs) and their genes *yprl* (Van Loon, 1999).

Structure

Anther-specific proteins form *Brassica napus* and *Arabidopsis* similar to β-1,3-glucanases but with a 114-amino acid carboxy-terminal extension and 37% identity to Tag1 might from a sixth class of β-1,3-glucanase. The only non-plant proteins reported so far to display similarity to plant PR-2-type proteins are fungal β-1,3-glucanase (Hird *et al.*, 1993). β-1,3-glucanases exist as multiple structural isoforms that differ in size, isoelectric point, primary structure, cellular localization, and pattern of regulation. The most detailed sequence information for these isoforms is available from cDNA and genomic clones of tobacco β-1,3-glucanase, which form a multigene family. Based on amino acid sequence identity, the various β-1,3-glucanase of the genus *Nicotiana* have been classified into three structural classes (Payne *et al.*, 1990).

The amino-terminal residue of the basic tobacco PR-2 most likely is a glutamine, an observation consistent with the presence of a 21-residue signal peptide in the preproteins. The three-dimensional structure of the barley β-1,3-glucanase has been determined by X-ray crystallography (Varghese *et al.*, 1994). The β-glycosyl residues are not bound equally tight to the individual sub sites and a slight repulsion rather than a binding is observed for the interaction between the third and the fourth sub site and the β-1,3 -glucan substrate. This might facilitate the hydrolytic cleavage catalyzed by amino acids located between the same third and fourth β-glycosyl-interacting sites.

The approximately 33 kDa class I enzymes (βGlu1) of *Nicotiana tabacum*, which constitute the PR-2e subgroup of the tobacco PRs are basic proteins localized in the cell vacuole. β-1,3-glucanase I is produced as a proprotein with an N-terminal hydrophobic signal peptide, which is cotranslationally removed, and a C-terminal

extension N-glycosylated at a single site. The proprotein is transported from the endoplasmic reticulum via the Golgi compartment to the vacuole where the C-terminal extension is removed to give the mature, approximately 33 kDa enzymes, which is not glycosylated (Sticher *et al.*, 1992).

Inspection of the amino acid sequences deduced from cDNAs or genes encoding PR-3-type proteins from different plants reveals that they all have in common a core domain harbouring the catalytic site and hence called the chitinolytic or catalytic domain. In addition, all PR-3-type proteins can be further divided in three subgroups, which are historically designated as class I, class II and class IV chitinases. Class IV chitinases mainly differ from the class I chitinases by four deletions, one occurring in the chitin-binding lectin domain, the other three in the catalytic domain. They also lack a carboxy-terminal propeptide (Collinge *et al.*, 1993).

The class II tobacco isoforms are at least 82% identical in amino acid sequence and differ from the class I enzymes at a minimum of 48.8% of the positions. The acidic, approximately 35 kDa, PR-2d is the sole representative of tobacco class III β-1,3-glucanase and differs in sequence at least by 43 % from the class I and class II enzymes. Two highly homologous cDNA clones for class III β-1,3-glucanase have been isolated from tomato plants infected with a viroid (Leubner-Metzger and Meins, 1999).

The PR-8-type protein family accommodates the chitinases, which were previously designated as class III chitinases (Van Loon *et al.*, 1994). This new nomenclature reflects the fact that PR-8-type chitinases are structurally unrelated to PR-3-type chitinases. Mature PR-8-type chitinases are monomeric proteins with a molecular weight of about 28 kDa and can be either acidic or basic. In most cases, PR-8-type chitinases have been reported to exhibit both chitinase and lysozyme activities but at least a sugar beet PR-8 chitinase exhibits endo- and exochitinase activity but no lysozyme activity (Nielsen *et al.*, 1993), indicating that bifunctional chitinase/ lysozyme activity is not a general property of this enzyme family.

A three-dimensional fold supposedly shared by all members of the PR-1-type protein family has been determined by NMR for the tomato P14a protein (Fernandez *et al.*, 1997). This protein features an α–β–α sandwich structure in which α-helices are tightly packed on both sides of a central β-sheet. The heptapeptide sequence HYTQ (hydrophobic residue) VW, which is conserved among plant and animal PR-1 like proteins, constitutes an α-helix that is largely buried inside the protein core and thus appears to play a structural role, possibly as a folding nucleus.

Occurrence

The occurrence of almost all types of PRs in various floral tissues also suggests specific physiological functions during flower development rather than a role in general defense against pathogen invasion. This notion is supported by the presence in floral organs of additional PR-like proteins, glucanases (Coté *et al.*, 1991) and thaumatin-like proteins (Richard *et al.*, 1992). In petunia flowers, chitinase activity is localized in the petals (about 15%) and stigma (about 85%). In the stigma it increases about five-fold following anther dehiscence, strongly suggesting that the chitinase has a specific function in reproduction (Neuhaus, 1999). Demonstration that the PR-2 family are β-1,3-endoglucanases and the PR-3,-4,-8, and -11 families consist of chitinases with or without lysozyme activity, immediately suggested that these PRs are directed against cell walls of fungi and bacteria. Homology of the thaumatin-like proteins of the PR-5 family with a bifunctional α-amylase/trypsin inhibitor from maize seeds seemed consistent with a role in protection against phytophagous insects. However, no proteinase inhibitor activity has been demonstrated for PR-5 proteins. Resistance to insect attack is taken to be conferred primarily by wound-inducible proteinase inhibitors, which have now been grouped into the family PR-6 (Heitz *et al.*, 1999).

A role of PRs as specific internal signal generating enzymes would be consistent both with their occurrence in specific organs and with their induction during the development and in response to stressful pathogen infections. The major chitinase of bean leaves first described by Boller *et al.* (1983) to be induced by ethylene and located in the vacuole, appears to be also induced in abscission zones at

the stem petiole-junction together with a PR-1-like protein, two isoforms of β-1,3-glucanase, other chitinases, and a thaumatin-like protein (Staehelin *et al.*, 1994). However, the natural substrates for chitinases in higher plant cell walls remain to be determined. PR-3-type proteins are monomeric enzymes ranging from about 25 to 35 kDa in size. They act as endochitinases and release chitooligosaccharides of varying degrees of polymerization, of which the smallest are usually chitobiose and chitotriose. A PR-3-type chitinase was first isolated from bean leaves but similar enzymes have now been purified from about 20 different monocot and dicot plants.

Elucidation of the biochemical properties of the major, pathogen-inducible PRs of tobacco and subsequent cloning of their cDNAs and/ or genes revealed that proteins with substantial similarity to the classical PRs, which are mostly acidic and extracellular proteins, the homologous counterparts are mostly basic and localized intracellularly in the vacuole. As far as it has been possible to deduce, they possess the same type of enzymatic activities, but their substrate specificity and specific activity may be rather different (Linhorst, 1991). Newly defined mRNAs (cDNAs) may be added to existing families when shown to be induced by pathogens or pathogen specific elicitors, like are the, thionins (Bohlmann, 1994) and defensins (Broekaert *et. al.*, 1995) both families are of small basic, cysteine-rich polypeptides, and qualify for inclusion as new families of PRs.

Biosynthesis

Comparison of the determined amino-terminus of a mature acidic PR-1 protein from tobacco and a basic PR-1 protein from tomato with the amino acid sequence derived form their respective cDNA sequences reveals that both proteins, as well as other PR-1-type proteins, are synthesized as preproteins consisting of an amino terminal hydrophobic signal peptide followed by the mature PR-1 domain. Some, but not all, basic PR-1-type proteins feature short (6 to 19 amino acids) carboxy-terminal segment that is absent in most other PR-1-type proteins. This carboxy-terminal extension may function as vacuolar targeting determinant in analogy to the proven role of the carboxy-terminal propeptide of some basic PR-2, PR-3 and PR-5 proteins (Melchers *et al.*, 1993).

Complementary DNA clones with sequences similar to tobacco *PR-1* sequences have been isolated from *Brassica napus*, *Solanum tuberosum*, *Medicago trunculata* and maize and have been purified from tomato, barley and *Arabidopsis* as well as from tobacco (Bryngelsson *et al.*, 1994). PR-1-type proteins have also shown significant homology to some proteins of non-plant origin.

PR-8-type chitinases display homology to concanavalin B, an enzymatically inactive protein from jack bean (*Canavalia ensiformis*). More intriguingly, plant PR-8-type proteins show sequence homology with a chitinase from baker's yeast (*Saccharomyces cerevisiae*). The overall identity between these proteins is 18% including 6 invariant cysteines, with an additional 43% of homologous amino acids in at least five out of the six aligned sequences (Broekaert *et al.*, 2000).

A single endochitinase from tobacco constitutes the class V chitinases, recently reclassified as PR-11 protein (Heitz *et al.*, 1994; Van Loon *et al.*, 1994). This enzyme is synthesized as a preprotein with an amino-terminal prepeptide and a carboxy-terminal propeptide loosely homologous to the tobacco basic β-1,3-glucanase carboxy-terminal extensions, although an N-glycosylation site is missing. The tobacco PR-11 chitinase shows significant homology to bacterial and nematode chitinases and to a lesser extent also to PR-8-type chitinases. Also remarkable is the fact that some fungal chitinases are more homologous to PR-8-type chitinases (e.g. Kuranda and Robbins, 1991), while other fungal chitinases are more related to PR-11-type chitinases (Hayes *et al.*, 1994).

Functional properties

PRs are, as such, a collective set of novel proteins which a plant produces in reaction to a pathogen mainly in incompatible interactions and thus impedes further pathogen progress. The "related situations" in which PRs were found to be induced, seem to prove the point. Application of chemicals that mimic the effect of pathogen infection or induced some aspects of the host response, as well as wound responses that give rise to proteins that are also induced during infections, can induce both PRs and acquired resistance. The occurrence of homologous PRs as small multigene

families in various plant species belonging to different families, their tissue-specificity during development and consistent localization in the apoplast as well as in the vacuolar compartment and their differential induction by endogenous and exogenous signaling compounds suggest that PRs may have important functions extending beyond their apparently limited role in plant defense.

During the hypersensitive reaction cellular damage and death is a major stress to the plant, as exemplified by high increases in abscisic acid and ethylene (Van Loon, 1997). It is possible, therefore, that PRs are stress proteins directed to alleviate harmful effects of cellular degradation products on thus far untouched neighboring cells. Both acidic and basic PRs may be induced by physiological necrosis or wounding or by high concentrations of ethylene (Brederode *et al.*, 1991). Such induction in the absence of pathogenic attack might be taken to indicate protection of cellular structures, either physically to stabilize sensitive membranes or macromolecules, or chemically to keep potentially harmful saprophytic microorganisms on tissue surfaces or in intercellular spaces in check. In virtually any natural stress condition e.g., heat, cold, drought, osmotic stress, water logging, anaerobiosis, metal toxicity, etc., plants are known to react by the synthesis of novel, and sometimes partly overlapping, sets of proteins (Wasternack and Parthier, 1997).

The various conditions under which PRs occur are reminiscent of those under which heat-shock proteins (HSP) are induced. These proteins are ubiquitous in living organisms and associated with the acquisition of thermotolerance to otherwise lethal temperatures, but a causal connection is not evident. Interestingly, the promoters of all three tobacco PR-1 genes that are expressed, as well as of a different type of PR in parsley, contain a heat shock regulatory element, but the proteins are not induced to detectable levels by heat shock. Nevertheless, PRs might have an analogous function, though quite different, chaperonin-like function unlike PRs, HSP are intercellular proteins that do not accumulate during heat shock. However, the specific occurrence of individual PRs in some floral organs, but not in others, points to other, more specific roles (Dutta and Muthukrishnan, 1999).

The relative ineffectivenes's of PRs in determining resistance to pathogens does not preclude an involvement in defense. As first proposed by Mauch and Staehelin (1989) acidic, extracellular PRs might be involved in recognition processes, releasing defense-activating signal molecules from the walls of invading pathogens. This would hold particularly for chitinases and glucanases that could liberate elicitor-type carbohydrate molecules from fungal and bacterial cell walls. Thus, a β–1,3–glucanase induced in soybean seedlings by infection or chemical stress releases elicitor-active fragments from cell wall preparations of the fungus *Phytophthora megasperma* f.sp. *glycinea*. Such elicitors cold help stimulate defense responses in adjacent cells and thus accelerate and enhance these reactions, as well as induce acquired resistance to further infection (Ham *et al.*, 1991).

PR-1 the most abundant of the PR proteins, is induced 10,000-fold in infected tissue and accumulates to 1 to 2 % of the total leaf protein. PR-1-type proteins are monomeric proteins of about 15 kDa with no known catalytic activity. They were first detected and purified from tobacco leaves infected with tobacco mosaic virus (Antoniw *et al.*, 1980). Tobacco PR-1-type proteins are encoded by a small gene family comprising at least 8 members. The acidic PR-1 proteins remain soluble at pH 3, whereas most other plant proteins are denatured under these conditions. The relative resistance to the action of endogenous and exogenous proteases, suggest that PR-proteins have a low turnover rate. The estimated half-life of the PR-1 proteins is 40 to 70 hours.

There are indications for the existence of special proteolytic enzymes that are responsible for the turnover of PR-proteins. The acidic *PR-1* genes do not encode a known targeting peptide sequence for vacuolar targeting. Therefore, the synthesis and accumulation within crystal idioblasts of the acidic PR-1 proteins, indicates that these cells sort the proteins in the unique manner Rodrigo *et al.*, (1991) described an extracellular aspartyl protease in tomato, which is able to degrade PR-proteins.

In other plants, extracellular proteins corresponding to the acidic PR-1 proteins of tobacco do not necessarily have a low pI. For example, the tomato P4 and P6 proteins purified from the apoplastic fluid are basic (Joosten *et al.*, 1990).

PR-1 proteins do not seem to function as antiviral factors despite the high production of PR-1a and -1b proteins after virus infection. Niderman *et al.* (1995) found that several PR-1-type proteins inhibit zoospore germination of the Oomycete *Phytophthora infestans*, the causal agent of potato late blight. In comparative tests, basic PR-1 proteins from either tobacco or tomato were significantly inhibitorier than acidic isoforms. Much investigation has not been done on the activity of PR-1-type proteins on other microorganisms, neither is much known on how they may affect growth processes of Oomycetes.

Plants contain several types of enzymes that have the ability to hydrolyze polysaccharides from microbial cell walls. Endo-β-1,3-glucanases, for instance, are enzymes that specifically hydrolyze β-1,3-ether linkages in β-1,3-glucans and β-1,6-glucans, resulting in a range of oligosaccharides down to disaccharides (Yoshikawa *et al.*, 1993). Most endo-β-1,3-glucanases are structurally related and form a family known as PR-2-type proteins which catalyze endo-type hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucans. The β-1,3-glucanses are abundant highly regulated enzymes widely distributed in seed plants (Høj and Fincher, 1995).

Although the major interest in β-1,3-glucanases stems from their possible role in the response of plants to microbial pathogens, there is strong evidence that these enzymes are also implicated in diverse physiological and developmental processes in the uninfected plant, including cell division, microsporogenesis (Bucciaglia and Smith, 1994), pollen germination and tube growth, fertilization (Ori *et al*, 1990) embryogenesis, fruit ripening, seed germination (Leubner-Metzger *et al.*, 1995), mobilization of storage reserves in the endosperm of cereal grains (Fincher, 1989), bud dormancy, and responses to wounding, cold, ozone and UV-B (Hincha *et al.*, 1997).

Basic glucanases belong to class I, whereas class II accommodates acidic β-1,3-glucanases including the two acidic 41 kDa stylar β-1,3-glucanase isoforms (Ori *et al.*, 1990) and class III β-1,3-glucanases harbor an acidic tobacco enzyme slightly

different from the class II enzyme. The products of three tandemly arranged *Arabidopsis* β-1,3-glucanase genes also fall into this class (Dong *et al.*, 1991).

Barley β-1,3- and structurally related β-1,3-1,4-glucanases are classified in a fourth class of PR-2-type proteins. *Tag1*, the PR-2-type protein, or a PR-like protein, specifically expressed in tobacco anthers forms the fifth class of β-1,3-glucanases (Bucciaglia and Smith, 1994). The tobacco PR-2 glucanases vary 250-fold in specific activity on laminarin and their relative activities on different substrates vary greatly, suggesting that their normal actions may be diverse. Expression studies of PR-2 in transgenic tobacco suggest that this protein functions developmentally in seed germination by weakening the endosperm, thus allowing the radicle to protrude (Vögeli-Lange *et al.*, 1994a).

There is considerable indirect evidence that, in analogy to tobacco class I chitinases and barley lectin, the C-terminal extension contains a signal for targeting to the vacuole. Results obtained with cultured tobacco cells provide strong evidence that vacuolar chitinases can be secreted into the medium via a novel pathway (Melchers *et al.*, 1993).

In contrast to β-1,3-glucanase I, the class II and III members of the PR-2 family are secreted into the extracellular space (Simmons, 1994). The tobacco class II β-1,3-glucanase PR-2a, PR-2b, PR-2c and the class III β-1,3-glucanase PR-2d, also known as PR-2, PR-N, PR-O, PR-Q′, respectively, are acidic proteins without the C-terminal extension present in the class I enzymes ranging in apparent size from approximately 34 to 36 kDa in denaturing gels (Van Loon *et al.*, 1994).

The specific enzymatic activities and substrate specificities of different β-1,3-glucanase vary considerably. The β-1,3-glucanase I and class II PR-2c appear to be 50 to 250 times more active in degrading the β-1,3-glucan substrate laminarin than the class II PR-2a and PR-2b and the class PR-2d enzymes (Linthorst, 1991).

Induction of β-1,3-glucanases I has been observed during germination. β-1,3-glucanases is exclusively induced in the micropylar region of the endosperm where the radicle will penetrate but prior to endosperm rupture. This induction of β-1,3-glucanase is not a classical defense response as chitinases and the known acidic II and III β-1,3-glucanases are not induced. So, it has been hypothesized that β-1,3-glucanase weakens the endosperm by digestion of cell wall material which promotes radicle protrusion to facilitate germination (Leubner-Metzger and Meins, 1999).

Gibberellins which can substitute for light in releasing dormancy in tobacco seeds, induces β-1,3-glucanase in the dark in association with germination. It is well established that 1,3;1,4-β-glucanases, which are structurally related to β-1,3-glucanase but differ in substrate specificity, hydrolyze the cell walls of the starchy endosperm during the germination of cereals (Høj and Fincher, 1995). Less is known about the function of cereal β-1,3-glucanase, which are present in ungerminated grains and rise markedly in concentration during germination. However it is proposed that high levels of β-1,3-glucanase are part of a strategy to protect the grain against microbial attack (Leubner-Metzger *et al.*, 1995). Cordero *et al.*, (1994) found that one β-1,3-glucanase isoforms and three chitinase isoforms are induced in germinating maize kernels infected by *Fusarium moniliforme*. In contrast, a second β-1,3-glucanase isoform is expressed in embryo and radicle tissues but is not induced by fungal infection. These findings support the view that cereal β-1,3-glucanase, in addition to their possible role in pathogen defense, might also be involved in embryogenesis and seed germination.

Later, it was shown that the PR-proteins include β-1,3-glucanase the PR-2 family and chitinase the PR-3 family. There is now compelling evidence that β-1,3-glucanase and chitinase, acting alone and particularly in combination, can help defend against fungal infection. It has been proposed that these glucanohydrolases act in at least two different ways: directly, by degrading the cell walls of the pathogen, and, indirectly by promoting the release of cell-wall derived materials that can act as elicitors of defense reactions (Boller, 1995). Several studies have been made in which different β-1,3-glucanase and chitinase isoforms were tested for *in vitro* antifungal

activity. In contrast, the class II β-1,3-glucanase PR-2a, PR-2b, and PR-2c did not exhibit antifungal activity alone or in any combinations tested. Similar studies with tomato β-1,3-glucanase and chitinase have shown that the vacuolar class I isoforms, but not the secreted class II isoforms, inhibit growth of *Alternaria solani*, *Trichoderma viride*, and *Phytophthora infestans* (Lawrence *et al.*, 1996; Kim and Hwang, 1997). Plant β-1,3-glucanase can release oligosaccharides from cell walls of the pathogens, which can then act as elicitors of defense reactions (Boller, 1995). This is well documented for interactions between soybean and the β-glucan elicitor from the pathogenic oomycete *Phytophthora megasperma* f.sp. *glycinea*. Following fungal attack, soybean β-1,3-glucanase releases β-glucans from fungal cell walls, which can then induce accumulation of the phytoalexin glyceollin.

The smallest β-glucan released with elicitor activity was a β-1,3-β-1,6-hepta-1,3-glucoside and the structural requirements for the elicitor activity of these oligosaccharides have been investigated. Umemoto *et al.* (1997) isolated cDNA for a β-glucan elicitor binding protein (GEPB), which is localized in the plasma membrane of soybean root cells. Expression of soybean GEPB gene has shown to confer β-glucan binding activity to *Escherichia coli* and to tobacco cells cultured in suspension, suggesting that GEPB might be an elicitor receptor. Soybean β-1,3-glucanase have been purified that are able to release active β-glucan elicitors from fungal cell walls. Ham *et al.* (1997) presented evidence that fungal pathogens secrete proteins that can selectively inhibit plant β-1,3-glucanase. Fungal pathogenesis appears to involve a complex interplay between host and pathogen β-1,3-glucanase. It is now recognized that species of higher plants produce a broad range of β-1,3-glucanase differing in primary structure, cellular localization, and catalytic activity. The available evidence suggests that different classes of β-1,3-glucanase have different functions in plant microbe interactions.

The activity of β-1,3-glucanase and β-D-glucosidases increased in the leaves of potato plants infected by species of the root parasite genus *Globodera* (Rahimi *et al.*, 1996). Enzyme assays revealed the appearance of both the extracellular and intracellularly targeted β-1,3-glucanase. The reactions of the cultivars to nematode

infection differed, indicating that different pathotypes of the same species or different species of potato cyst nematode elicited the appearance of different classes of β-1,3-glucanase. Kim and Hwang (1997) isolated a basic β-1,3-glucanase with inhibitory activity against *Phytophthora capsici* and found a close serological relationship between the pepper 34 kDa β-1,3-glucanase and tomato β-1,3-glucanase. A basic β-1,3-glucanase (34 kDa) induced in pepper (*Capsicum annuum*) stems by mercuric chloride treatment was purified by CM-cellulose cation exchange chromatography. The 34 kDa β-1,3-glucanase inhibited hyphal growth of the chitin-negative fungus *Phytophthora capsici*, but did not show antifungal activity against the chitin-containing fungi *Alternaria mali, Colletotrichum gloeosporioides Magnaporthe grisea,* and *Fusarium oxysporium* f.sp. *cucumerinum.* Treatment with higher amounts of the β-1,3-glucanase caused lysis of the hyphal and zoospore cells of *P. capsici.* Moreover the 34 kDa β-1,3-glucanase acted synergistically with a pepper basic 32 kDa chitinase in inhibiting hyphal growth of *F. oxysporium* f.sp. *cucumerinum* and *P. capsici.*

Ji and Kuc (1997), found that non-host resistance to *Colletotrichum lagenarium* in pumpkin and squash is not primarily associated with β-1,3-glucanase and chitinase activities. Disease symptoms of anthracnose were observed in cucumber, pumpkin and squash after infiltrating leaves with a conidial suspension of *Colletotrichum lagenarium*, but symptoms developed only in cucumber when droplets of the conidial suspension were applied to the leaf surface. There was no difference in the germination of conidia or in appressorium formation on leaf surfaces of cucumber, pumpkin or squash plants; however, penetration was markedly reduced into pumpkin and squash with or without systemic acquired resistance (SAR) and into cucumber with SAR. Little β-1,3-glucanase and chitinase activities were detected in challenged pumpkin and squash leaves without symptoms even after 5 days after inoculation in leaf surfaces. However, the enzymes were detected in pumpkin and squash leaves with symptoms, and activities of the enzymes were greater than in cucumber. These results suggest that β-1,3-glucanase and chitinase activities are not primarily initial defense compounds associated with non-host resistance of pumpkin and squash to *C. lagenarium.*

The localization of systemically induced chitinase activity was examined (Boller and Metraux, 1988) in the second leaves of cucumber plants that had been infected on the first leaf with tobacco necrosis virus. Leaf pieces were infiltrated with a buffer sodium-chloride solution, and the intercellular fluid was subsequently extracted by centrifugation. chitinase had a much higher specific activity in extracts of the intercellular fluid than in leaf homogenates. The specific activity of chitinase in protoplasts obtained from the leaves was only 5% of that in tissue homogenates. These data indicate that most of the chitinase activity is located in the extracellular space. Few of the inducible acidic PRs associated with SAR have been shown to possess significant anti-pathogenic activity. It could be that PRs make cells less conducive, but any such evidence is lacking. Moreover, PRs do not determine the resistance response, and at most have only an accessory role (Van Loon, 1997).

Characterization

The high resistance of PR-proteins to acidic pH and to proteolytic attack appears to be good adaptation to the conditions of the extracellular environment. Acidic PR-1 proteins were detected in extracellular spaces and xylem elements of TMV-infected tobacco leaves, using biochemical and immuno-localization experiments (Dumas *et al.*, 1988). In addition, (Dixon *et al.*,1991) described the accumulation of PR-1a, -1b, and -1c after TMV infection of *N. tabacum* Xanthi in the central vacuoles of specialized leaf cells, known as crystal idioblasts. Genomic clones and cDNAs corresponding to basic proteins, which are approximately 65% similar to the acidic PR-1a, -1b and -1c proteins, have also been characterized from the TMV-infected leaves (Cornelissen *et al.*, 1987; Payne, *et al.*, 1989). Apart from tobacco PR-1 proteins exist in other organism too. Immunoblot analysis using antibodies against the acidic tobacco PR-1b revealed the existence of the cross-reacting proteins in a number of infected dicots and monocots, including tomato, potato, cowpea, maize, sunflower, barley, *Gomphrena globosa, Chenopodium amaranticolor* and *Solanum demissum.* So far there are indications for the two basic PR-1 proteins in tobacco although hybridization studies revealed that the genome contains at least eight (pseudo) genes. (Cornelissen *et al.*, 1987) Antibodies against the tobacco acidic PR-1 proteins did not react with the basic proteins.

β-1,3-glucanases were first purified from leaves of bean and tobacco in the early 1970s (Abeles *et al.*, 1971). Kauffmann *et al.* (1987) reported that four pathogenesis related proteins induced in tobacco upon infection were in fact β-1,3-glucanase. Comparison of data from barley β-1,3-glucanases purified from various monocot and dicot plant species indicates that they are all monomers with a molecular weight of about 30 kDa (Boller, 1988). These proteins and their homologs are now known as PR-2-type proteins (Van Loon *et al.*, 1994). Phylogenetic analysis of PR-2-type proteins derived from cDNA or genes isolated from several plants yields a dendogram with at least five clusters of related proteins (Bucciaglia and Smith, 1994).

The induction of β-1,3-glucanase as a part of the hypersensitive reaction is a stereotypic response i.e., the pattern of induction is similar for viral, bacterial and fungal pathogens. β-1,3-glucanase show developmental regulation in response to treatment with hormones or infection with pathogens. More recently, specific β-1,3-glucanase proteins have been measured immunologically and their mRNAs have been measured semi-quantitatively by RNA-blot hybridization. In a limited number of cases, regulation of transcription has been studied using plants transformed with Gus reporter genes under the control of the promoter region of β-1,3-glucanase genes. β-1,3-glucanase accumulates at high concentrations in the roots and in lower leaves of mature, healthy tobacco plants. The β-1,3-glucanase content of leaves decreases toward the top of the plant. Within leaves, they are localized in the vacuole of epidermal cells (Keefe *et al.*, 1990).

Further, Metraux *et al.* (1988), found a PR in both the infected and uninfected leaves of cucumber plants inoculated on the first true leaf with a fungal, a bacterial or a virus pathogen. This host-coded protein was detected up to 5 leaves above the infected leaf. The protein was purified from the intercellular fluid by ion-exchange chromatography and by high performance liquid chromatography on ion-exchange and phenyl-sepharose columns. The purified PR was shown to be a chitinase with a molecular mass of 28000 Da as determined by SDS-polyacrylamide gel electrophoresis and by gel filtration.

Biochemical and immunological characterization of tomato PR proteins was elucidated by Fischer *et al.* (1989). Acid soluble apoplastic proteins were isolated from leaves of *Lycopersicon esculentum* (Mill) plants infected by *Phytophthora infestans* and separated by two-dimensional gel electrophoresis. Two groups of proteins with either basic (pI $\geq$ 8) or acidic (pI $\leq$ 6) pIs were distinguished. Characterization of defense related proteins that exhibit properties of chitinase and β-1,3-glucanase has also been demonstrated in the wax of "Carnauba" (*Copernica cerifera*), an economically important palm tree, by Cruz *et al.* (2002).

Induction

Usually the most effective inducers of PRs are pathogens that cause a necrotic reaction; the largest amounts of PRs occur in tissues adjacent to the necrotic areas. However, some pathogens can induce large amounts of PR without causing necrosis, for example potato virus Y infection of tobacco induces the systemic production of high concentrations of PRs 1a, 1b, 1c and 2 (Kassanis *et al*, 1974) and *Burkholderia solanacearum* induces large amounts of PR1 proteins in infiltrated tobacco leaf, neither producing any necrotic symptoms. The suppression of mannitol induces PR1 accumulation in tobacco by antibiotic (White *et al.*, 1996).

Many plant species react to treatment with the stress hormone ethylene with induction of β-1,3-glucanase I activity, protein and mRNA in leaves (Beffa *et al.*, 1995). Although ethylene increases the β-1,3-glucanase I content of epidermal cells slightly, its inductive effect is most pronounced on mesophyll cells of the tobacco leaf. Treatment of plants with ethephon results in no detectable induction, or very weak induction of class II and class III β-1,3-glucanase in leaves of tobacco and tomato.

Tobacco cultivars carrying the dominant *N* gene from *N. glutinosa* show a local lesion, HR response to TMV rather than systemic symptoms of mosaic disease. These plants also show decreased disease symptoms in response to secondary infection with certain other viral, bacterial and fungal pathogens. This is an example of a type of induced long lasting, broad-spectrum resistance called systemic acquired

resistance (SAR). Systemic accumulation of salicylic acid (SA) is associated with the HR of tobacco, *Arabidopsis thaliana*, and certain other plants (Delaney *et al.*, 1995). Treatment of mature wild-type tobacco plants with SA strongly induces accumulation of mRNAs of PR related class II and III β-1,3-glucanase and certain other PRs. While SA is probably not the long distance systemic signal for SAR activation, it is required for transduction of this signal in leaves distal to the primary infection site.

Investigations on the stress response in *Brassica* species susceptible and resistant to *Phoma lingam* was carried out by Dixelus (1994). Experiments were set up to study the effects of fungal isolates of different virulence on both a resistant and a susceptible *Brassica* host species. Two isolates of *Phoma lingam*, the fungal toxin sirodesmin PL, and salicylic acid were used to induce stress in plantlets of *Brassica napus* and *B. nigra*, which are susceptible and resistant to the fungal pathogen, respectively, *B. nigra* showed a very rapid response to antisera raised against PR-2, PR-Q and PR-S in tobacco. In contrast, in *B. napus* stressed by the fungus, sirodesmin PL or salicylic, the production of the three PR-proteins was delayed or absent.

Spraying plants with a solution of salicylic acid mainly induces the acidic PR-1 and acidic β-1,3-glucanase genes (Bol *et al.*, 1990). Salicylic acid is a naturally occurring compound in tobacco plants and the levels of endogenous salicylic acid rise after TMV infection, not only in the infected leaves but also in the uninfected parts of the plant, which show induced resistance (Malamy *et al.*, 1990). In many plant species, resistance can be induced against a range of pathogens by pre-treating the plant with certain chemicals or compounds of biological origin, ranging from inorganic salts to cell wall fractions from fungi. Pre-inoculation with pathogens or non-pathogens stimulate the same signal transduction pathways. Resistance can be local and restricted to the treated tissue or be induced systemically. The induction of resistance usually coincides with the accumulation of pathogenesis related proteins and their transcripts in resistant tissue (Kuc, 1982). Of various chemicals and biological compounds known to induce resistance in different species of plants, only 2,6-dichloroisonicotinic acid (INA) possessed the ability to induce resistance in sugar beet. Nielsen *et al.* (1994b) demonstrated that repeated spraying with low

concentrations of INA during the week prior to challenge inoculation with the fungus induced complete local and systemic resistance.

Eleven acid soluble proteins with apparent molecular masses ranging from 13-82 kD increased in tomato (*Lycopersicon esculentum* Mill.) leaves infected by *Phytophthora infestans* or *Fulvia fulva*. The most prominent changes in the protein pattern were also detectable in the untreated leaves of infected plants indicating systemic effects of the infection. Similar changes in the proteins were induced by moderate irradiations of the leaves with UV-light (254 nm) and by injecting the leaves with indole-3-acetic acid, 2-chloroethyl- phosphoric acid, ethephon, fusicoccin or an elicitor preparation from *Phytophthora megasperma* f.sp. *glycinea*. Acetyl salicylic acid, aspirin, kinetin and abscisic acid did not induce detectable changes in protein pattern nor did they induce resistance. Some of the changes in specific proteins might be useful markers of induced resistance (Christ and Mösinger, 1989).

Biosynthesis of PR-proteins in sugar beet has been intensively investigated both on plants infected by pathogens and treated with synthetic inducers of SAR: salicylic acid and its function derivates 2,6-dichloroisonicotinic acid (INA) and benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH). BTH was shown to induce resistance to a number of fungal and viral pathogens, e.g. *Arabidopsis*, wheat, tobacco. Analysis of extracellular fluid isolated from BTH-treated sugar beet leaves revealed the accumulation of acidic and basic proteins displaying both chitinase and β-1,3-glucanase activities indicating the ability of BTH to activate defence reactions in sugar beet (Burketova *et al.*, 2003). However, in contrast there was no increase in accumulation of transcripts encoding three chitinase isozymes (including Ch4) and β-1,3-glucanase in sugar beet leaves following the treatment with INA, compound similar to BTH, even though the INA pretreatment completely inhibited the development of *Cercospora beticola* (Nielsen *et al.*, 1994a). Recently, the BTH capability of inducing SAR to root pathogens was shown on cucumber plants against *Pythium* damping-off as well as to *Phytophthora* root rot (Ali *et al.*, 2000) and even to root-parasitic weed *Orobanche cumana* and root-knot nematodes, suggesting that BTH-induced or potentiated defence mechanism might be of more general character. Similarly, the cross-activity of defence responses against diverse pathogens has been

demonstrated on rhizomania-diseased sugar beet and *Heterodera schachii* root nematodes

Activation of natural weapons before infection, called systemic acquired resistance (SAR) is initiated by pathogens, pathogen- or pathogen-derived elicitors, as well as a number of chemical compounds. Among the main defence genes, which are switched on in response to pathogen infection, belong those encoding PRs. The association of PRs with SAR, but not with ISR, has led to the hypothesis that accumulation of PRs is not a pre-requisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon, 1997). SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRs become detectable in the infected plants. The observations indicate that individual PRs are induced to various extents by these different signals. Consequently, the mixture of signals released or produced upon microbial stimulation appears to determine the magnitude of the plant's response and its effectiveness to inhibit further infection. A salicylate-inducible *PR-10* gene subclass from apple, designated *Apa*, was highly activated by wounding and ethephon as well as differentially expressed during infection with a compatible and incompatible race of *Venturia inaequalis* (Poupard *et al.*, 2003).

Induction of PRs has since been found to be invariably linked in necrotizing infections giving rise to SAR, and has been taken as a marker of the induced state (Ward *et al.*, 1991; Uknes *et al.*, 1992; Kessman *et al.*, 1994). This notion has been reinforced by the characterization in *Arabidopsis* of mutants that either are comprised in both the production of PRs and the induction of SAR (*npr1*; Cao *et al.*, 1994, *nim1*; Delaney *et al.*, 1995), or are constitutive expressors of PR genes as well as SAR (*cpr1*; Bowling *et al.*, 1994).

Systemic acquired resistance (SAR) of plants induced upon infection with a necrotizing pathogen, is characterized by an enhanced capacity to induce cellular defence responses to subsequent pathogen attack. Pre-treating the first leaves of cucumber plants with the synthetic SAR activator acibenzolar-S-methyl (*S*-methylbenzol[1,2,3] thiadiazole-7-carbothiate) protects whole plants from infection

with the virulent fungal pathogen *Colletotrichum arbiculare* (Cools and Ishii, 2002). Nakashita *et al.* (2002) elucidated the SAR signaling pathway in tobacco using peobenazole and its active metabolite 1,2-benzisothiazole-1,1-dioxide which induce SAR in *Arabidopsis* through salicylic acid accumulation.

Immunolocalization

Localization of the major, acidic PRs in the intercellular space of the leaf seems to guarantee contact with invading fungi or bacteria before these are able to penetrate. In localization studies *in planta*, labelling for β-1,3-glucanases and chitinases was especially pronounced over fungal cell walls confirming their role in plant defence. Most of the investigations were devoted to leaf tissues. In roots, expression of defence genes was studied on infection by pathogens, arbuscular mycorrhizal fungi, antagonistic fungus *Trichoderma harzianum* or non-pathogenic bacterium *Pseudomonas fluorescens* and differences in the expression of distinct classes of chitinases and β-1,3-glucanases were reported in dependence on the particular microbial inducer. Similar results of localization of PR-3 proteins have been reported in potato leaves infected by *Phytophthora infestans* (Garcia-Garcia *et al.*, 1994). Subcellular localization of PR-1 proteins was studied in roots of resistant *Nicotiana tabacum* cv. *Xanthi* uninfected or infected *in vitro* by the black root rot fungus *Chalara elegans*, using polyclonal or monoclonal antibodies raised against PR-1 protein. In healthy tobacco roots, the PR-1 proteins were found to be present in low amounts in intercellular space material, over cell walls and over secondary thickening of xylem vessels. All these cell compartments were significantly enriched in the PR-1 proteins in infected tobacco root tissues. Their accumulation over the cell walls of inter- and intracellular hyphae of *C. elegans* colonizing tobacco roots may reflect an eventual role of these proteins, in association with other PRs like β-1,3-glucanases and chitinases in directly hindering hyphal growth of the pathogen (Tahiri-Alaoui *et al.*, 1993).

Two antisera raised against acidic β-1,3-glucanase and acidic chitinase from tobacco were used to investigate the subcellular localization of the two enzymes in *Fusarium culmorum*-infected wheat spike by means of the immunogold labelling technique (Kang and Buchenauer, 2002). The studies demonstrated that the

distribution of β-1,3-glucanase and chitinase were localized mainly in the cell walls of different tissues including the lemma, ovary and rachis of the wheat spike, while the cytoplasm and the organelles of cells in these tissues showed almost no labelling. However, the accumulation of β-1,3-glucanase and chitinase in the infected wheat spikes differed distinctly between resistant and susceptible wheat cultivars.

Immunohistological localization of chitinase and β-1,3-glucanase in rhizomania-diseased and benzothiadiazole treated sugar roots (Burketova *et al.*, 2003) revealed accumulation of plant basic β-1,3-glucanase and basic class IV chitinase cell walls and extracellular spaces. The deposition of the enzymes was predominantly found in endodermis, cortical cells adjacent to endodermis, and xylem vessels. Similar to the pathogens, benzothiadiazole (BTH) induced deposition of both chitinase and β-1,3-glucanase systemically in cortex and endodermis cells.

In *in situ* localization studies of chitinase mRNA and protein in compatible and incompatible interaction triggered by *Phytophthora capsici* in pepper stems Lee *et al.* (2000) showed that induction of chitinase mRNA (*CAChi2*) mRNA started as early as 6 h after inoculation and gradually increased in the incompatible interaction of pepper stems with *P. capsici*. Another interesting finding of the authors was specific immunogold labelling of chitinase on the cell wall of the oomycete as early as 24 h after inoculation. In particular they showed that numerous gold particles were deposited on the cell wall of *P. capsici* with a predominant accumulation over areas showing signs of degradation in the incompatible interaction and that healthy pepper stem tissues were nearly free of labelling.

Transgenics

Plant protection is a major challenge to agriculture worldwide. One of the effective strategies for disease resistance in plants has been the incorporation of disease resistant genes into commercially acceptable cultivars. The most attractive initial candidates for manipulation of the single gene defense mechanisms approach are genes encoding chitinase and β-1,3-glucanase because these two enzymes hydrolyze chitin and β-1,3glucans which are structural components of the cell walls

of several fungi. chitinase gene from *Rhizopus oligosporus* has been shown to operate as an antifungal system in transgenic tobacco. Transgenic cucumber harboring the rice chitinase genes exhibited enhanced resistance against gray mold, *Botrytis cinerea.* While it is clear that it is possible in several cases to alter the expression of chitinase transgenes to generate plants with increased resistance to the pathogen, it is not clear whether constitutively expressed chitinase alone is responsible for the reduction of disease symptoms as observed in the case of tobacco and canola. Introduction of bacterial chitinase gene from *Serratia marcescens* in transgenic tobacco cells showed up to an eightfold increase in amount of chitinase protein in the plants and conferred resistance to *Rhizoctonia solani.* Expression of β-1,3-glucanase in transgenic tobacco plants was shown to result in enhanced resistance to *Alternaria alternata.* Combined expression of PR-2 (β-1,3-glucanase) and PR-3 (chitinase) gives effective protection against fungal infection as they have been shown to act synergistically.

Major interest has been devoted to plant hydrolases, β-1,3-glucanases (E.C.3.2.1.39) (PR-2) and chitinases (EC 3.2.1.14) (PR-3), as they are capable of cleaving fungal cell walls resulting in pathogen growth inhibition (Neuhaus, 1999), and moreover, the products of the hydrolysis can act as elicitors of further defence responses (Boller *et al.*, 1995). Both β-1,3-glucanases and chitinases are highly abundant proteins in plant involved in diverse physiological and developmental processes. They can act either alone or in combination strengthening their antifungal activity. Their accumulation is not restricted only to resistant plants but is often observed in compatible plant-pathogen interactions or even non-pathogenic combination.

Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom expression, consistent with a role of PRs in the expression of acquired resistance (Ryals *et al.*, 1994). Significant suppression of disease symptoms caused by the soil-borne fungus *Rhizoctonia solani*was demonstrated in tobacco or canola expressing vacuolar class I chitinase from bean (Broglie *et al.*, 1991), the basic tobacco chitinase PR-3c (Vierheilig *et al.*, 1993), tobacco or cucumber PR-8 (Lawton *et al.*, 1993), or the (class II) barley chitinase (Jach *et al.*, 1995), but enhanced chitinase levels caused no significant protection

against *Cercospora nicotianae* (Zhu *et al.*, 1994) or *Fusarium oxysporum* (Van den Elden *et al.*, 1993).

Antifungal activity of chitinases can be synergistically enhanced by β-1,3-glucanses, both *in vitro* and *in vivo*. Thus co-expression of chitinase and glucanase genes in tobacco enhanced resistance against *C. nicotianae* (Jach *et al.*, 1995). In tobacco simultaneous expression of the basic tobacco chitinase PR-3d and glucanase PR-2e afforded substantial protection against *F. oxysporum* f.sp. *lycopersici*, whereas transgenic plants expressing either one of these genes were not protected (Jongedijk *et al.*, 1995). An additional 'SAR gene' in tobacco, SAR 8.2. when expressed constitutively in transgenic tobacco, was also found to reduce disease caused by *P. parasitica*, but the protein has not been characterized (Van Loon, 1997).

Proteins serologically related to the tobacco PR-1 proteins were not only detected in tomato, but in a variety of other plant species as well, including dicotyledonous plants such as cowpea and potato, and monocotyledonous species like maize and barley (Nassuth and Sänger, 1996). Genes encoding PR-1-like proteins were isolated from *Arabidopsis thaliana* (Metzler *et al.*, 1991) and maize (Casacuberta *et al.*, 1991). The maize PR-1-like mRNA is expressed in germinating seeds and further induced by infection with the fungus *Fusarium monoliforme*.

Transgenic plants with chimeric genes containing promoter fragments from a chitinase (class III) gene from *Arabidopsis* fused to the GUS reporter gene were studied by Samac and Shah (1991) and was found that the proximal 192 bp from the transcription start site contains elements necessary for salicylate-inducible expression. Naderi and Berger (1997) have observed the presence of potato virus Y coat protein (PVY-CP) inside chloroplasts of infected tobacco cells. Induction of symptoms reminiscent of virus infection was observed only in transgenic plants accumulating CP in the chloroplast but not in the cytoplasm. These results suggested that PVY-CP plays a role in symptom development. Plants with high levels of CP in their chloroplasts were more susceptible to PVY, suggesting that there was a host response to the presence of PVY-CP in the chloroplasts.

There is strong evidence that expression of β-1,3-glucanase transgenes alone or in combination with chitinase transgenes regulated by the strong CaMV ^{35}S RNA promoter can reduce the susceptibility of plants to infection by certain fungi. Transgenic tobacco plants expressing a soybean β-1,3-glucanase can elicitor releasing β-1,3-glucanase or the tobacco class II β-1,3-glucanase PR-2b show reduced symptoms when infected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotianae* and *Perenospora tabacina* (Lusso and Kuc, 1996). β-1,3-glucans are the major components of the cell walls of oomycetes, a group of fungi that do not contain chitin. Wubben *et al.* (1996) reported on the tissue-specific expression of genes encoding the hydrolytic enzymes, β-1,3-glucanase and chitinase, as studied by means of *in situ* hybridization. Only temporal differences in gene transcript accumulation were observed for each isoform studied. Expression of the acidic chitinase gene was observed primarily near leaf vascular tissue. Expression of the basic chitinase and basic and acidic β-1,3-glucanase genes was less confined to particular tissues. No preferential accumulation of gene transcripts in tissue near penetrating hyphae was observed in compatible or incompatible interactions. Roulin *et al.*, (1997) studied the expression of β-1,3-glucanase genes in leaves of near-isogenic resistant and susceptible barley lines infected with the leaf scald fungus, *Rhynchosporium secalis*. The difference in expression of β-1,3-glucanase between the resistant backcross lines clearly showed that there are physiologically distinct modes of resistance to the scald fungus in barley, and that at least one of these is accompanied by β-1,3-glucanase induction.

Activation of natural weapons before infection, called systemic acquired resistance (SAR) is initiated by pathogens, pathogen- or pathogen-derived elicitors, as well as a number of chemical compounds. Among the main defence genes, which are switched on in response to pathogen infection, belong those encoding PRs. The association of PRs with SAR, but not with ISR, has led to the hypothesis that accumulation of PRs is not a pre-requisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon and Van Strien, 1997). SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRs become detectable in the

infected plants. The observations indicate that individual PRs are induced to various extents by these different signals. Consequently, the mixture of signals released or produced upon microbial stimulation appears to determine the magnitude of the plant's response and its effectiveness to inhibit further infection.

In *Arabidopsis* SA-dependent expression of PR-1, PR-2 and PR-5 is required for increased protection against the biotrophic fungus *Perenospora parasitica*, whereas SA-independent but JA-dependent induction of the plant defensin gene *pdf1·2*, as well as of PR-3 and PR-4, is associated with the induced resistance against the necrotrophic fungi *A. brassicicola* (Pennickx *et al.*, 1996), *Botrytis cinerea* (Thomma *et al.*, 1998) and *Fusarium oxysporium* f.sp. *matthiolae* (Epple *et al.*, 1997; Epple *et al.*, 1998). These results suggest that the SA- and JA-dependent defense pathways in *Arabidopsis* contribute to resistance against distinct microbial pathogens. As a result, PRs and similarly induced antimicrobial proteins appear to contribute differentially to the induced resistance against different pathogens.

Klessig *et al.* (2000) have identified several potential components of the SA signaling pathway, including the members of the TGA/OBF family family of bZIP transcription factors. These bZIP factors physically interact with NPR1 and bind the SA-responsive element in promoters of several defense genes, such as the PR-1 gene. Additionally, they showed that nitric oxide might activate PR-1 expression via an NO-dependent pathway. Barwe *et al.* (2002) demonstrated that zeatin induces the accumulation of β-1,3-glucanase gene transcripts. However, the accumulation of transcription was blocked by protein kinase inhibitor staurosporine, the protein synthesis inhibitor cyclohexamide, and the calcium channel blocker verapamil.

Gene-specific expression studies (Ko *et al.*, 2003) and characterization of a tissue-specific and developmentally regulated β-1,3-glucanase gene family including two newly isolated basic β-1,3-glucanase genes, *PpGns2* and PpGns3, linked in tandem array has in *Prunus persica* revealed that alternate members of members of β-1,3-glucanase genes were expressed at different stages of development. Transgenic tomato plants overexpressing the prosystemin transgene accumulate a soluble 87 kDa cystatin constitutively (Siqueira-Junior *et al.*, 2003). This protein of the PR-6 family

has been demonstrated to have a role in defense response against pests and pathogens. Proteinase inhibitors, have also been identified in rice compatible host-blast pathogen interactions (Agrawal *et al.*, 2002)

Ergon *et al.* (1998) opined that cold hardening in wheat had a conditioning effect on snow mold (*Microdochium nivale*) induced expression of PR-proteins. Gaudet *et al.* (2003) demonstrated that the temporal expression of cold-induced, plant defense-related transcripts in winter wheat is differentially regulated among genotypes and during different developmental stages, and are the first to implicate LTPs in resistance induced at low temperatures during natural acclimation of winter wheat. Two PR-5-like genes were isolated (Campos *et al.*, 2002) from black nightshade (*Solanum nigrum* L. var. *americanum*) genome. Kruger *et al.* (2003) demonstrated that transcription of defense response genes chitinase IIb, PAL and peroxidase is induced by the barley powdery mildew fungus and is only indirectly modulated by R genes.

Despite major advances in the field of plant disease resistance, the precise molecular mechanisms of plant pathogen recognition and the detailed dissection of R-gene mediated signaling networks remain elusive. In the years ahead, new genomic and proteomic technologies will assist in the identification of signaling components and in the investigation of the biochemical functions of R-proteins and other signaling molecules. Beyond gene identification, proteomic approaches will provide insights into the biochemical properties of proteins involved in disease resistance (Martin *et al.*, 2003).