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Summary — The hypersensitive reaction to a pathogen is one of the most efficient defense mechanisms in nature and leads to the induction of numerous plant genes encoding defense proteins. These proteins include: 1) structural proteins that are incorporated into the extracellular matrix and participate in the confinement of the pathogen; 2) enzymes of secondary metabolism, for instance those of the biosynthesis of plant antibiotics; 3) pathogenesis-related (PR) proteins which represent major quantitative changes in soluble protein during the defense response. The PRs have typical physicochemical properties that enable them to resist to acidic p*H* and proteolytic cleavage and thus survive in the harsh environments where they occur: vacuolar compartment or cell wall or intercellular spaces. Since the discovery of the first PRs in tobacco many other similar proteins have been isolated from tobacco but also from other plant species, including dicots and monocots, the widest range being characterized from hypersensitively reacting tobacco. Based first on serological properties and later on sequence data, the tobacco PRs have been classified in five major groups. Group PR-1 contains the first discovered PRs of 15–17 kDa molecular mass, whose biological activity is still unknown, but some members have been shown recently to have antifungal activity. Group PR-2 contains three structurally distinct classes of 1,3-β-glucanases, with acidic and basic counterparts, with dramatically different specific activity towards linear 1,3-β-glucans and with different substrate specificity. Group PR-3 consists of various chitinases-lysozymes that belong to three distinct classes, are vacuolar or extracellular, and exhibit differential chitinase and lysozyme activities. Some of them, either alone or in combination with 1,3-β-glucanases, have been shown to be antifungal *in vitro* and *in vivo* (transgenic plants), probably by hydrolysing their substrates as structural components in the fungal cell wall. Group PR-4 is the less studied, and in tobacco contains four members of 13–14.5 kDa of unknown activity and function. Group PR-5 contains acidic-neutral and very basic members with extracellular and vacuolar localization, respectively, and all members show sequence similarity to the sweet-tasting protein thaumatin. Several members of the PR-5 group from tobacco and other plant species were shown to display significant *in vitro* activity of inhibiting hyphal growth or spore germination of various fungi probably by a membrane permeabilizing mechanism. We have isolated several other tobacco PRs that cannot be classified in these five major groups: two hydrolases with α-amylase activity, two inhibitors of microbial proteases, two peroxidases, two basic proteins of 16 and 45 kDa of unknown activity. In conclusion, among the 33 isolated tobacco PRs, 31 have been characterized and 25 are members of five major groups also found in other plant species. Most of the PRs (groups PR-1, PR-2, PR-3, PR-5, α-amylases, inhibitors of microbial proteases) can be considered as direct antimicrobial defense proteins or enzymes. Some of them might also be indirectly antimicrobial, for instance PRs-2 and PRs-3 by releasing elicitor-active oligosaccharides, and peroxidases by catalysing cross-linking of macromolecules in the cell wall.

hypersensitive response / chitinase-lysozyme / 1,3-β-glucanase / proteinase inhibitor / membrane permeabilization

Introduction

Pathogenesis-related (PR) proteins were first detected in the early 1970s [1, 2] in tobacco leaves reacting hypersensitively to tobacco mosaic virus (TMV). This is the biological system that we are also using and from which the highest number of PR-proteins have been characterized so far.

The hypersensitive reaction to pathogen attack is considered as a very strong defense response induced in plants by the pathogen itself. It follows the phenomenon of gene-for-gene complementarity [3–6] between plant and pathogen. In the presence of dominant, matching alleles of a pathogen avirulence gene and of a plant disease resistance gene, recognition

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occurs and the plant responds to infection by producing the hypersensitive response [7–9]. Necrotic lesions are formed around the initial sites of pathogenic attack. The pathogen will be restricted to a zone of cells surrounding the necrotic lesions, as a result of a very intense defense response that is induced in this ring of cells [10]. In the case of the hypersensitive response of tobacco to TMV, the zones of highly-induced defense responses can easily be detected under UV light as rings of cells exhibiting bright blue fluorescence and surrounding the necrotic lesions. These cells have accumulated derivatives of the phenylpropanoid pathway [11-13], some of which are fluorescent, and thus represent good indicators of defense responsive cells. Other low molecular mass components accumulate equally well in these cells and have antimicrobial activity, the so-called 'phytoalexins' [14–17]. But many other changes occur in the same cells [10, 18, 19]. For instance, cell wall thickening and reinforcement by deposition of various macromolecules such as carbohydrates (callose), proteins [20] and hydroxyproline-rich glycoproteins [21], aromatic polymers such as lignin, lignin-like material or other yet undefined cell wall bound phenolics [22, 23]. Another important change which occurs in the fluorescent cells is the production of the numerous PR-proteins which, after a few days post-inoculation, may account for 10% of the content of the leaves in soluble proteins [24–29].

Some of these changes are strictly localized around the infection sites, for instance the accumulation of fluorescent phenylpropanoid metabolites of tobacco [12] or generally in most plant species the production of low molecular mass antimicrobial substances [10, 17]. However, some of the changes are also induced at distance from the infection sites and even in uninoculated and uninfected parts of partially-infected plants, though to a much lower extent than locally. This is the case for some of the PR-proteins, whose production in uninoculated tobacco leaves represents 5–10% of their production in inoculated hypersensitively reacting leaves. Even though this 'systemic' defense response is of much lower cellular intensity than the local response it still represents a massive amplification in the response since it concerns the whole plant. Furthermore, this systemic defense response is accompanied by the phenomenon of 'systemic acquired resistance'. If part of a plant has already reacted hypersensitively to a pathogen, the uninoculated parts of this plant develop an increased state of resistance evidenced by smaller lesions and greater restriction of the infection upon challenge inoculation by the same or any other unrelated but necrotizing pathogen [30–37]. Since the PR-proteins are induced in parallel with the appearance of systemic acquired resistance they are believed to participate in its efficiency, but a causal relationship has still not been clearly proven. They represent also very interesting and sensitive markers for the search of the signals involved that are moving from the primary sites of necrotizing infection to distant sites where both systemic acquired resistance and PR-proteins are induced [38].

Occurrence, induction and general properties of PR-proteins

Since the discovery of the first four PR-proteins in tobacco [1, 2], many other proteins with similar physicochemical and induction properties have been isolated from tobacco (see below) as well as from many other plant species, including dicots and monocots.

It has been reported that PRs are produced during the response of plants to infection by viruses, viroids, bacteria or fungi (for reviews see [26, 39–43]). Very interestingly, they are produced constitutively in interspecific hybrids which exhibit high constitutive resistance to viral infection [44]. However, at least some members of the different PR families have been found to be induced under treatments with chemicals such as polyacrylic acid [45], amino acid derivatives [46], heavy metal salts [47, 48], aspirin and salicylic acid [40, 42, 49–54] and air pollutants [55, 56]. Some PRs are also induced by treatments with phytohormones. Even though cases of induction by auxins [57, 58] or cytokinins [59, 60] are known, the pattern of induction by these two hormones is unclear, except under conditions of strong disturbance in the balance between them which lead to high induction of various families of PRs [61–65]. Some PRs are induced under osmotic stress or salt stress conditions [66, 67] with a possible involvement of abscisic acid [68]. Another phytohormone, ethylene, considered as a 'stress' hormone, is a potent inducer of PR proteins [58–60, 69–77]. PR production has also been shown to be developmentally regulated in healthy plants since high levels of some PRs have been reported in roots and senescent leaves [60, 62, 64, 66, 78, 79], and during flowering [80–84].

Since PRs are induced under so many different conditions where pathogens are not involved, should they still be called 'pathogenesis-related'? In fact, the widest range of PRs in a given plant species is induced in response to pathogens (see below) and, therefore, this name appears still justified. Furthermore, it is known that during response of plants to pathogens, and particularly in necrotic infections, metabolic alterations include the production of the stress hormones ethylene and possibly abscisic acid, the synthesis and migration of the strong PR inducer salicylic acid [51–53, 85–87] and changes in the balance between auxins and cytokinins. As mentioned above, all these individual changes have the potential

to induce the production of a given set of PRs, and, hence, it is not surprising that the response to necrotic infections which produces a combination of these inducers is accompanied by the induction of the widest array of PRs.

PR-proteins display very characteristic physicochemical properties which aid in their detection and isolation: 1) they are very stable at low pH and remain soluble (for instance in an extraction buffer of pH 2.8) whereas most other plant proteins are denatured; 2) they are relatively resistant to the action of proteolytic enzymes of endogenous but also exogenous origin; 3) they are monomers (with a few exceptions, see below) of rather low molecular mass (8–50 kDa); 4) they are localized in compartments such as the vacuole, the cell wall and/or the apoplast. Properties 1), 2), and 4) are tightly related, *ie* the high level of resistance of PRs to acidic pH and to protease action appear to be well adapted to these harsh environments where they occur. Resistance to proteases also suggests that the PRs have a low turnover. There has been a report, however, indicating a degradation of PRs by an extracellular aspartyl protease which might be responsible for PR turnover in tomato [88].

One can wonder whether the conditions of induction or these physicochemical properties should be taken into account to define a protein as pathogen-related. In the opinion of the authors, rather than giving priority to the induction conditions which are common to many other defense-related proteins and enzymes, one should consider high resistance to very acidic medium and to proteases as an additional property of PRs, since they enable these proteins to survive in very hostile environments to which they are targeted: the vacuolar compartment, the intercellular spaces and the cell wall.

Classification of PR-proteins

The first four discovered PRs [1, 2] could easily be detected because of their very high mobility in native basic polyacrylamide gels and because they migrated to a zone of the gel containing very few other cellular proteins. Other, less mobile PRs were present in the same gels but could not be resolved from the numerous proteins with similar mobilities. The procedure of extraction at low pH introduced in the late 1970s eliminated most of the less mobile proteins, and a comparison of the gels loaded with extracts from tobacco leaves reacting hypersensitively to TMV or from healthy leaves revealed six other bands of PRs [24]. These 10 major acidic tobacco PRs were well resolved upon electrophoresis on these native basic gels and their nomenclature was based on their relative electrophoretic mobility. They have been referred to as PR-1a, -1b, -1c, -2, -N, -O, -P, -Q, -R and -S in order of decreasing mobility (fig 1) [89]. PR-1a, -1b and -1c were studied extensively and shown to be serologically related. We purified to homogeneity the seven other PRs [27, 90] and demonstrated that they were members of three other distinct serological families containing PR-2, -N and -O, PR-P and -Q, PR-R and -S, respectively [90]. The availability of these proteins in their native form enabled us to assign biological functions to five of these proteins which have remained mysterious for 15 years: we demonstrated that PR-2, -N and -O had endo-1,3-β-glucanase activity [91] and that the major proteins PR-P and -Q had endo-chitinase activity [92]. This finding could then be extended to other plant species from which PRs were also identified as endo-1,3-β-glucanases or endo-chitinases [93–98].

In the late 1980s, proteins similar to those of tobacco were detected or characterized in many plant species, their cDNAs and gene(s) cloned, so that many sequence data became available. They confirmed sequence similarities between the PRs mentioned above having chitinase or glucanase activity

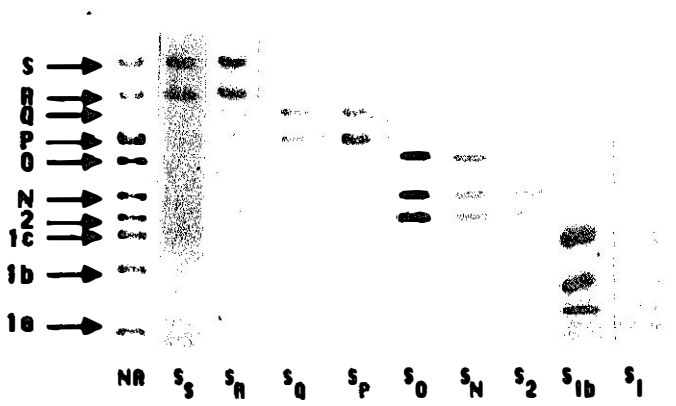

Fig 1. Serological relationships between the 10 acidic extracellular PR-proteins initially detected in tobacco by their resistance to acidic pH and to protease cleavage [24]. A mixture of the 10 proteins purified according to [90, 99] were submitted to slab gel electrophoresis under non-denaturing conditions, as described in [92]. After electrophoretic transfer onto nitrocellulose sheets, the proteins were stained with amido black (lane NA) or immunodetected with sera raised against proteins PR-S, -R, -Q, -P, -O, -N, -2 and -1b (lanes S_S, S_R, S_Q, S_P, S_O, S_N, S_2 and S_{1b}, respectively), or incubated with a preimmune serum (lane S_I). Note that the proteins are well resolved from each other under the native conditions of electrophoresis used and that, in addition to the known PR-1 serological group containing three proteins [26, 89] there are three other distinct serological groups including two, two and three proteins, respectively.

Table I. The tobacco PR-1 group: antifungal proteins. Proteins PR-1a, -1b and -1c were isolated according to [27, 90]. Protein PR-1g has been isolated recently by the authors (unpublished results) and its amino acid sequence is almost identical to that deduced from previously described cDNA and genomic clones [109, 110]. The nomenclature is that proposed in [29, 111]. Similar boxes indicate demonstrated serological relationships. Molecular mass values are either deduced from corresponding cloned cDNA or calculated from relative mobility upon electrophoresis of the proteins under denaturing conditions. Values of p*I* are calculated from the amino acid sequences or elution pH have been measured in chromatofocusing experiments. The localization is referred to 'ext' for extracellular and 'int' for intracellular occurrence of the proteins.

Name	*Molecular mass (kDa)*	*Elution pH (chromatofocusing)*	*Localization*	*Biological activity*
1a	15.5	<7	ext	antifungal
1b	15.5	4.5	ext	antifungal
1c	15	4.7	ext	?
1g	17	>7	ext	?

and well known constitutive plant chitinases and glucanases, respectively. However, they usually did not give any clue to the biological activity or function of the other families of PRs.

We have extended our studies in tobacco by both molecular biology and biochemical approaches. Concerning the biochemistry, we used a combination of conventional and HPLC means of chromatography involving a cascade of steps based on various physicochemical properties of the proteins [99]. These steps include cation or anion exchange chromatography, chromatofocusing, hydrophobic interaction and molecular sieving. By combining these steps we were able to isolate up to now from tobacco, that is still considered as a prototype system for PR-proteins, more than 30 PR-proteins. Isolation of these proteins in their native form allowed us to raise antibodies, but the major advantage of having them in the native form was the possibility to determine and compare their specific biological activities.

Among the newly-isolated proteins some are related to the 10 acidic extracellular proteins already described in tobacco. Some are obviously members of new families in tobacco, but are related to proteins of other plant species. At the 3rd International Workshop on PR-proteins (Arolla, Switzerland, August 16–20, 1992) it was agreed that to enable an easier comparison of related PR-proteins members between different plant species, a common classification and way of nomenclature should be used. It was particularly agreed and recognized that five families or groups of PR-proteins have been found in most studied plant species and, thus, appear to be ubiquitous.

The following paragraphs will describe these different groups and their possible role in defense, taking the tobacco PRs as the model system.

The PR-1 group

The present stage of knowledge concerning tobacco proteins of group 1 is summarized in table I. Tobacco acidic PRs of group 1 (PR-1a, -1b and -1c) were the subject of most of the earlier work. They were the first to be purified [27, 89, 100] and were shown to belong to a serological group in tobacco and to be serologically related to PRs from other plant species [94, 101–103]. cDNA clones encoding the acidic tobacco PRs of group 1 were isolated from cDNA libraries derived from mRNA extracted from leaves reacting hypersensitively to TMV [104–108]. It was shown that these three proteins have sequence similarity higher than 90% and that their increased synthesis is under transcriptional control. They are encoded by a small family of at least eight genes [109] and several of these genes have been isolated from genomic libraries (for reviews see [40, 42]). cDNAs and genomic clones corresponding to a basic protein which has approximately 65% similarity to PR-1a, -1b and -1c have also been characterized from TMV-infected leaves [109, 110]. Using the antibodies raised against tobacco acidic PR-1 proteins, it was not possible to detect basic PR-1 protein(s). Recently, we were able to isolate a basic 17 kDa protein and partial microsequencing indicated almost complete identity to the protein sequences deduced from the cDNA and genomic clones (Fritig *et al*, unpublished). According to the rules proposed by a nomenclature committee for PR-proteins, this basic member should be called PR-1g [111].

Comparison of the amino acid sequences of all known PRs-1 with those available in data banks did not give any clue about the potential biological activity or function of these proteins [105, 112]. Since they were the first discovered PRs in response to viruses, they have been believed to participate in resistance to viruses or in their localization. Consistent with this hypothesis several treatments that induced PRs-1 in tobacco were also efficient inducers of resistance to viruses. Another strong argument was provided by the interspecific hybrids of *Nicotiana glutinosa* x *Nicotiana debneyi* that produced constitutively PRs-1 and were highly resistant to TMV [44]. This question was addressed further by producing transgenic tobacco plants expressing PR-1a or PR-1b genes constitutively [113, 114]. In transformed plants the protein encoded by the chimeric gene was produced and targeted to the extracellular spaces, as expected. However, these plants did not show higher

levels of resistance to virus infection, and apparently represent an argument that breaks the correlation between high levels of resistance to virus and high levels of PRs-1.

Some data are already available, suggesting that some PR-1 members have antifungal activity. It has been shown recently [115] that three members of the PR-1 group from tomato had antifungal activity in *in vitro* assays against *Phytophthora infestans*. In the same assays, PR-1a and PR-1b from tobacco showed lower but still significant activity of inhibiting *Phytophthora infestans* (Niderman and Fritig, unpublished). This activity observed *in vitro* for PR-1a is consistent with recent results obtained *in vivo* by the group of John Ryals [38]: they observed that transgenic tobacco plants expressing constitutively high levels of PR-1a exhibited a significant protection from infection by *Peronospora tabacina* that causes the blue mold disease of tobacco.

It will be now interesting to assay other PRs-1 of various plant species for antimicrobial activity and to investigate the molecular mechanism(s) involved.

The PR-2 (1,3-β-glucanase) and PR-3 (chitinase) groups

Since the discovery that major tobacco PR-proteins had endo-chitinase [92] and endo-1,3-β-glucanase activity [91], the literature on plant chitinases and 1,3-β-glucanases has grown to such an extent that a comprehensive analysis is beyond the limitations of this review which will not deal with the structure and the regulation of their genes, but will focus merely on their biological activities and their role in defense.

Properties and biological activities of PRs-2 (1,3-β-glucanases)

Many 1,3-β-glucanases have been purified and characterized (for a review see [116]). Like most of the PR-proteins, they usually are monomers with a molecular mass in the 25–35-kDa range. Most are endoglucanases, producing oligomers of chain lengths of 2-6 glucose units from the classical substrate laminarin, an almost unbranched 1,3-β-glucan, or from other 1,3-β-glucans. Many glucanases from various plant species have been cloned and sequenced (for a review see [117]). On the basis of a comparison of the deduced amino acid sequences of tobacco 1,3-β-glucanases, they have been grouped into three classes [118–120].

From tobacco we have isolated five distinct members (table II): four acidic enzymes and a major basic enzyme (that consists perhaps of several closely related isoforms, as suggested by two-dimensional polyacrylamide gel electrophoresis [67]). The latter is a class I enzyme, PR-2, -N and -O are of class II, and another extracellular enzyme, PR-Q' has to be considered in the separate class III according to sequence data [118]. They all are serologically related [90, 91]. A protein called O', migrating like PR-O in native basic gels [99], did not cross-react with anti-glucanase antibodies, has a dimeric structure, and was found to possess no glucanase activity after several successive chromatographic steps aimed to remove any contamination by PR-O. The acidic enzymes are extracellular [90, 91] whereas the basic counterpart occurs in the vacuoles [72, 121]. Another 1,3-β-glucanase called sp41 has been found in large amounts in specific flower organs [81]. Unlike other PRs of group 2, sp41 is glycosylated and has not been found after TMV infection.

We have measured the specific activity of these enzymes towards laminarin as the substrate, and, surprisingly found pronounced differences, exceeding two orders of magnitude (table II). It is noteworthy that there is no correlation between the level of sequence similarity and the relative catalytic activity of the enzymes: PR-proteins 2 and N show 99% amino acid similarity but N was found five times

Table II. The tobacco PR-2 group: 1,3-β-glucanases. The proteins were isolated according to [90, 91, 99]. The different classes are based on differences in amino acid sequence as described in [117–120]. Specific enzymatic activity has been measured with laminarin as the substrate as described in [91]. Relative specific activity is expressed as the percentage of the highest specific activity. For all other items, see legend to table I.

Name	*Class*	*Molecular mass (kDa)*	*Elution pH (chromatofocusing)*	*Localization*	*Relative specific activities (%)*
2	II	31	4.4	ext	0.4
N	II	33	4.7	ext	1.8
O	II	35	4.8	ext	85
Q'	III	35	5.3	ext	3.8
glucb	I	33	>7	int	100

Table III. The tobacco PR-3 group: chitinases – lysozymes. Proteins chi 34, chi 32, P and Q were isolated according to [90, 92, 99]. Proteins chi 28, lys 28a, lys b1 and lys b2 have been isolated recently by the authors (unpublished results). The different classes are based on differences in amino acid sequence as described in [117, 123, 131–133]. Chitinase and lysozyme activities were measured as described in [92] and [126], respectively. For all other items, see legend to table I.

Name	*Class*	*Molecular mass (kDa)*	*Isoelectric point (elution pH)*	*Localization*	*Relative specific activities (%)* *Chitinase*	*Lysozyme*
chi 34	Ia	34	>7	int	100	10
chi 32	Ia	32	>7	int	100	10
chi 28	Ib	28	>7	ext	30	10
P	IIa	27	(5.3)	ext	20	0
Q	IIa	28	(5.8)	ext	20	0
lys 28a	III	28	4.4	ext	10	0
lys b1	III	28	8.3	ext	10	80
lys b2	III	28	8.3	ext	20	100

more active than 2; likewise PR-O is 93% similar to PR-2 in amino acid sequence but is 250 times more active. It has an activity similar to that of the basic glucanase, even though the two amino acid sequences are very different so that they fall into distinct classes (table II). This raises the problem of differences in substrate specificity between glucanases of a given plant species and also between glucanases of different plant species.

Clear evidence for such differences in substrate specificity was obtained in a collaborative work with P Albersheim and A Darvill (Complex Carbohydrate Research Center, Athens, Georgia, USA). They purified to homogeneity a soybean 1,3-β-glucanase [122] that shows also high specific activity towards laminarin, like PR-proteins O and gluc b of tobacco (table II). Preparations of pure soybean glucanase and of the most active tobacco glucanases were adjusted to the same activity towards laminarin and faced with another substrate consisting of a preparation of cell walls from the fungus *Phytophthora megasperma*, a pathogen of soybean. Very striking differences in activity were observed for the different glucanases: the tobacco enzymes had no significant activity towards this substrate, whereas the soybean glucanase was very active (Fritig *et al*, unpublished). In conclusion, it appears that 1,3-β-glucanases may differ very much in specific activity towards a given substrate and more generally in substrate specificity.

Properties and biological activities of PRs-3 (chitinases-lysozymes)

A large number of chitinases have been purified and characterized (for reviews see [116, 123]). Like the glucanases they are usually monomers of 25–35-kDa molecular mass. Most plant chitinases characterized so far are endochitinases, producing chito-oligosaccharides of 2–6 N-acetylglucosamine units. Many purified plant endochitinases also possess some lysozyme activity, *ie* they can hydrolyse β-1,4-linkages between N-acetylmuramic acid and N-acetylglucosamine in the bacterial peptidoglycan [116, 124–127]. Likewise, most plant lysozymes show significant levels of chitinase activity [126–128]. Some chitinases also act as chitosanases but, in addition, specific chitosanases are induced in plants in response to pathogens [129].

In response to TMV infection, tobacco plants produce PR-P and PR-Q as the major acidic PRs. We showed [92] that they hydrolysed efficiently chitin, a polymer of β-1,4-N-acetylglucosamine, that is a major component of the exoskeleton of insects and of the cell wall of most fungi. This was very surprising since the inducing pathogen, TMV, does not contain chitin. Furthermore, two other chitinases of basic isoelectric point (chi 32 and chi 34) were also found induced by TMV infection. Chi 32 and chi 34 are the most active enzymes and account for about 70% of the total chitinase activity in TMV-infected tobacco extracts. These forms have been detected in other situations, for instance in tobacco tissue and protoplast cultures [64, 67] and, like other basic members of other groups of PRs, in roots and old leaves of tobacco [64]. The basic chitinases chi 32 and chi 34 have also lysozyme activity, in contrast to chitinases P and Q (table III). Recently a third basic chitinase, chi 28, closely related to chi 32 and 34 has been isolated (Stintzi *et al*, unpublished). Furthermore, two other basic proteins, lys b1 and lys b2, have been isolated and demonstrated to have high lysozyme activity on *Micrococcus luteus* cells (Stintzi *et al*, unpublished) and a significant chitinase activity. Antibodies raised against lys b1 recognized lys b2 and

a novel acidic protein called lys 28a. The latter was purified and, surprisingly showed no significant lysozyme activity in the assay with *Micrococcus luteus* cells. Since at the pH of incubation there could be repulsion effects of negative charges between the enzyme and the substrate, we used another classical substrate of lysozymes, 4-methylumbelliferyl-chitotriose, and found that all three lysozymes had high activity towards this substrate (Stintzi *et al*, unpublished).

Concerning the localization of the different members of tobacco chitinases-lysozymes, some are vacuolar proteins while others are extracellular. Till recently, all acidic tobacco proteins had been found to be extracellular and all their basic counterparts localized in the vacuoles [40]. Now, in addition to PR-1g (table I) which is an extracellular basic protein, table III reveals three other basic proteins that are targeted to the extracellular spaces: chi 28, lys b1 and lys b2.

Comparison of deduced amino acid sequences of cDNA and genomic clones indicates that there are at least three classes of chitinases in tobacco [123]. Class I contains the basic chitinases with a N-terminal cysteine-rich domain of approximately 40 amino acids and a highly conserved main structure, separated by a variable hinge region. Class II chitinases include PR-P and PR-Q and lack the N-terminal cysteine-rich domain but have a high amino acid sequence identity to the main structure of class I chitinases [130, 225, 226]. In contrast, class III chitinases of tobacco have no similarity with class I and class II but are very similar to a cucumber chitinase having lysozyme activity [131–133].

Like for glucanases, chitinase structures appear very conserved in the plant kingdom. This was evidenced by serological studies [48, 94–97, 134, 135] and from comparison of the decoded cDNA or gene sequences (for reviews see [117, 123]). Thus, class I, class II and class III chitinases have been found in various plant species. Recent studies have shown that a short C-terminal extension of a few amino acids in a tobacco class I chitinase was both necessary and sufficient for vacuolar localization [136]. Such sequences are also found in other vacuolar chitinases from tobacco, bean, potato, poplar and *Arabidopsis* [74, 137-140] and these chitinases are designated as class Ia to distinguish them from class Ib found in *Brassica napus*, pea, rice and barley [123, 141-143] and lacking this C-terminal extension, suggesting extracellular localization. In tobacco, chi 28 has an N-terminal sequence identical to chi 32 and chi 34 (Stintzi *et al*, unpublished); it is extracellular since it probably lacks also the C-terminal end present in chi 32 and chi 34.

A fourth distinct class of chitinases, class IV, still not detected in tobacco, has been proposed to include chitinases present in sugar beet, rape and bean [123, 144]. These class IV chitinases contain a cysteine-rich domain and a conserved main structure which are similar to those of class I chitinases but smaller due to several deletions.

The antimicrobial activities of glycanhydrolases of groups PR-2 and PR-3

It was suggested already in 1971 [145] that chitinases and 1,3-β-glucanases may function as a defense against fungal pathogens, but support for this suggestion came much later when several plant chitinases were purified to homogeneity and shown to digest cell walls of pathogenic fungi [70, 146] and when some of the extracellularly targeted PR-proteins were found to possess chitinase [92] or 1,3-β-glucanase [91] activity.

Direct antimicrobial activity of chitinases and 1,3-β-glucanases

Antifungal activities *in vitro* of plant chitinases and 1,3-β-glucanases have been studied with various bioassays (for reviews see [116, 147]). Antifungal activity of plant chitinases was first shown in a bioassay on agar plates with a saprophyte, *Trichoderma viride* [148]. A similar bioassay was used by several groups to demonstrate a significant inhibition of growth of rapidly growing saprophytes [124, 134, 149], but the results were rather disappointing with a number of phytopathogenic fungi examined, with some exceptions [150]. Using a modification of this bioassay with small filter disks laid on the agar and enzyme solutions applied to the disks it was shown that chitinases and 1,3-β-glucanases purified from elicited pea pods, taken individually, were inhibitory only in exceptional cases to fungal growth around the filter disks [151]. But combinations of the two purified enzymes inhibited growth of most of the fungi studied and more or less mimicked the antifungal activity observed with the crude pea pod extracts [151]. Using a bioassay in microtiter plates that allows to observe the growth of fungi in liquid media and the same plant hydrolases, it was found that inhibition of growth of *Nectria haematococca* was only transient [152], suggesting that the fungus apparently had the capacity to adapt to the chitinases and the 1,3-β-glucanases in its growth medium. However, the bioassay confirmed the synergistic effects of chitinase and 1,3-β-glucanase, that were also found for several of the tobacco hydrolases [153, 154]. A detailed analysis of the *in vitro* antifungal activities of individual hydrolases and of various combinations was made. Class I chitinases (table III) and class I glucanase (table II) were the most active in the lysis of hyphal tips and in inhibiting the growth of *Fusarium solani* germlings. The class II isoforms had no effect if assayed individually. This again underlines the striking differences in substrate

specificity between glucanases (for instance PR-O and gluc b of table II) even if they exhibit similar capacities of hydrolysing laminarin. Furthermore, class II chitinases showed limited inhibitory activity in any combination. Finally the most efficient system was shown to be a combination of class I chitinases and class I glucanases that provided strong synergistic effects. Synergistic antifungal activities were also observed for combinations of basic chitinases and basic glucanases purified from sugar beet and assayed against *Trichoderma viride* and *Cercospora beticola* [155]. Since chitin and 1,3-β-glucan fibres are synthesized simultaneously in the apex of growing hyphae of filamentous fungi, and since these polysaccharides may cross-link and be overlaid with other material in cell wall distant from the apex, these results observed *in vitro* are not really surprising: nascent chitin of the hyphal apex is the most accessible to hydrolysis by chitinase [123] and the effectiveness of a hydrolase may depend on the simultaneous action of another one to hydrolyse mixed chitin-glucan fibres.

What about antimicrobial activity *in vivo* of glycan-hydrolases? First there are several lines of evidence obtained from immunocytochemical approaches that hydrolases are indeed produced in the vicinity of infection sites and accumulate around fungal cell walls *in planta* [156–158]. Furthermore, there is generally a spatio-temporal regulation so that glucanases and chitinases are produced simultaneously at the same sites, suggesting that synergistic effects required for optimal antifungal activity *in vitro* are likely to occur *in vivo*.

Since many chitinase and 1,3-β-glucanase cDNAs or genes have been cloned, the prospects for genetically engineering plants with improved resistance by transformation with chitinase or/and glucanase genes are reasonably good, and this approach is now being utilized in many laboratories all over the world (for reviews see [159, 160]). Several successful cases have already been reported. Tobacco and canola plants constitutively expressing high levels of a class I bean chitinase were more resistant against the root pathogen, *Rhizoctonia solani* [150, 161], whose sensitivity *in vitro* to this chitinase was well documented by an immunocytochemical approach showing a progressive breakdown of labelled chitin [162]. Transgenic tobacco expressing high levels of either the tobacco class I basic chitinase, the tobacco acidic class III chitinase or the cucumber class III chitinase showed significant resistance against disease caused by *Rhizoctonia solani* [38]. Reduction in disease symptoms caused by the same fungus was also reported in transgenic tobacco constitutively expressing a chitinase of bacterial origin [163]. In contrast, *Nicotiana sylvestris* transgenic plants expressing high levels of the tobacco class I chitinase showed unaltered susceptibility to the leaf pathogen, *Cercospora nicotianae*. It is possible that the highly expressed chitinase, since it is located intracellularly, did not come to contact with the intercellular developing fungus. Since many fungi grow intercellularly, the manipulated enzymes should be targeted to the extracellular spaces. Such attempts are already under way, and the most potent antifungal tobacco hydrolases of class I have been modified at their C-terminus to be targeted to the extracellular spaces of transgenic plants. It was found [153] that the extracellular washing fluids indeed contained the modified proteins that had retained full antifungal activity when assayed individually *in vitro* and their synergistic potential when used in combination. Experiments are now in progress to see whether targeting of the tobacco class I hydrolases to the extracellular space is an effective way of increasing fungal resistance. There is an example, however, that removal of the targeting sequence of the class I tobacco chitinase suppressed resistance against *Rhizoctonia solani* in transgenic plants [164]. There is also a report of an antibacterial resistance obtained in transgenic potato expressing constitutively phage T4 lysozyme [165]. It will be interesting to test antibacterial resistance by constitutive expression of plant lysozyme genes, for instance those of class III chitinases-lysozymes.

In conclusion, direct antifungal activities of plant chitinases and 1,3-β-glucanases have been clearly demonstrated with various bioassays. It is very likely that plants use induction of these hydrolases as an efficient defense response. Further results obtained from transgenic plants expressing high constitutive levels of hydrolases have to be awaited before it can be concluded that the strategy is valuable and mimics, at least partially, a pathogen-induced defense response. In this respect, the observations in [152] are important showing that *in vitro* fungi are only temporarily inhibited by chitinases and 1,3-β-glucanases and can adapt to even high levels of these hydrolases. Thus, a constitutively expressed high level may not be very effective in genetically-engineered defense, and it would be preferable to increase their levels suddenly in the vicinity of approaching hyphae. In natural induced defense, such as the hypersensitive reaction, levels of hydrolases are highly induced within a short time and operate in two successive lines: a first line of extracellularly-targeted enzymes and a second line of the vacuolar and very active enzymes released during hypersensitive cell death and collapse [71].

Indirect antimicrobial activity of chitinases and glucanases

It has been known for some time that plant chitinases and 1,3-β-glucanases have a potential to partially

degrade fungal cell walls [70, 146, 151]. The products released are oligosaccharides, some of which have been shown to be perceived by the plant cell as signals, so-called 'elicitors', and to induce active defense responses in the absence of any infection (for reviews see [16, 167, 168]). Indeed, chitin and chitosan oligomers and 1,3-β-glucans with some branching have been shown to induce a set of metabolic alterations typical of defense responses [16, 167–171]. The biologically active oligochitins and oligochitosans are thought to be released during the plant-pathogen interaction by the action of plant chitinases and chitosanases [168, 170]. This might lead to an amplification of the defense responses, some of the induced proteins being again chitinases [171]. Concerning the occurrence and activity of glucan elicitors (for a review see [167]), many cases have probably to be re-examined since the discovery that the activity of fungal elicitor fractions believed to contain mainly glucans was due to proteinaceous or glycoprotein elicitors [172–175, 227]. Indeed the elicitors active on soybean are glucans derived from the fungal cell wall and their structure has been clearly established [176, 177]. It has also been shown that soybean endo-1,3-β-glucanases were indeed able to release elicitor active fragments from the fungal cell wall [122,178–180].

In conclusion, plant chitinases and 1,3-β-glucanases are likely to play a dual role: they are directly antimicrobial by hydrolysing structural components of the cell wall of microbes, furthermore they are also indirectly antimicrobial by releasing oligosaccharides that are active elicitors of defense responses and, in turn, may be responsible for the amplification of the defense responses [10, 18, 167].

The PR-4 group

In tobacco four proteins of the PR-4 group have been isolated from TMV-infected leaves and named r1, r2, s1, s2 [181]. They are all acidic proteins (table IV), with yet no known basic counterpart, and migrate on the native basic gel system (see fig 1) in the vicinity of acidic PRs-5. In fact the couples r1/r2, s1/s2 migrate close to PR-R and PR-S, respectively. However, in denaturing gels, PRs of group 4 migrate as small 13–14.5-kDa proteins and are clearly distinguishable from other PRs [181]. They all are extracellular and serologically related to each other but do not show a serological relationship to other PRs or PR-groups. Primary structure deduced from cDNA clones is known for two of these proteins [182, 183] and indicates 50% sequence similarity to Win1 and Win2 that are induced in potato in response to wounding. They show also significant similarity to the C-terminal sequence of hevein, a protein from latex of *Hevea brasiliensis*. Nothing is known about the biological function or activity of these proteins which have not been studied extensively. They represent also the less ubiquitous of the five PR-groups since only one similar protein has been described so far, a 15-kDa tomato protein [184].

The PR-5 group: 'thaumatin-like' proteins

In tobacco Samsun NN this group includes two almost neutral proteins named R and S (table V). These extracellular proteins were among the 10 initially detected on the native basic gel system [24] and we demonstrated [90, 181] that they belonged to a distinct serological group (see also fig 1). They correspond probably to proteins R-minor and R-major found by others [185] in the cultivar Xanthi nc of *Nicotiana tabacum*. Two basic serologically-related counterparts which accumulate also in infected leaves (table V) have been identified as osmotins [67, 186]. Osmotin had been previously found to accumulate in tobacco cells in response to salt stress [187]. The basic proteins of group PR-5 are localized in the vacuolar compartment [67, 187] whereas the slightly acidic-neutral members R and S are apoplastic [188, 189]. cDNAs have been cloned which correspond to acidic [190, 191] and basic [192] tobacco PRs-5. There is more

Table IV. The tobacco PR-4 group: 'win-like' proteins. The proteins were isolated according to [181]. For all other items, see legend to table I.

Name	*Molecular mass (kDa)*	*Elution pH (chromato-focusing)*	*Localization*	*Biological activity*
r2	13	7.0	ext	?
r1	14.5	7.0	ext	?
s2	13	7.4	ext	?
s1	14.5	7.4	ext	?

Table V. The tobacco PR-5 group: 'thaumatin-like' proteins. The proteins were isolated as described in [181, 186]. For all other items, see legend to table I.

Name	*Molecular mass (kDa)*	*Elution pH (chromato-focusing)*	*Localization*	*Biological activity*
R	24	6.9	ext	?
S	24	7.5	ext	antifungal
n-osmotin	22	>7	int	?
osmotin	22	>7	int	antifungal

Table VI. Anti-fungal activity of PR-5 proteins estimated from inhibition of spore germination (S) or of hyphal growth (H).

Proteins	*Fungus*		*Effects*	*References*
	Genus/species	*Class*		
Osmotin (tobacco)	*Phytophthora infestans*	Oomycetes	S	[200]
	Candida albicans	Hyphomycetes	S	[209]
	Neurospora crassa	Ascomycetes	S	[209]
	Trichoderma reesei	Hyphomycetes	S	[209]
PR-S (tobacco)	*Cercospora beticola*	Hyphomycetes	H	[209]
AP 24 (tomato)	*Phytophthora infestans*	Oomycetes	S	[200]
Thaumatin *(T daniellii)*	*Candida albicans*	Hyphomycetes	H	[199]
Zeamatin (corn)	*Candida albicans*	Hyphomycetes	S	[210]
	Trichoderma reesei	Hyphomycetes	S	[210]
	Neurospora crassa	Ascomycetes	S	[210]
	Fusarium oxysporum	Hyphomycetes	H	[224]
	Alternaria solani	Ascomycetes	H	[224]
Trimatin (wheat)	*Candida albicans*	Hyphomycetes	S	[199]
	Trichoderma reesei	Hyphomycetes	H, S	[199]
	Neurospora crassa	Ascomycetes	H, S	[199]
Protein R (barley)	*Candida albicans*	Hyphomycetes	S	[197]
	Trichoderma viride	Hyphomycetes	S	[197]
	Fusarium oxysporum	Hyphomycetes	S	[197]
Protein S (barley)	*Candida albicans*	Hyphomycetes	S	[197]
	Trichoderma viride	Hyphomycetes	S	[197]
	Fusarium oxysporum	Hyphomycetes	S	[197]

than 90% sequence identity between PR-R and PR-S and more than 60% between the acidic and the basic counterparts. There is also 60% sequence similarity between the acidic PRs-5 and thaumatin, a sweet-tasting protein extracted from the african shrub, *Thaumatococcus daniellii*; therefore, the proteins of group PR-5 are also called the 'thaumatin-like PRs'. Thaumatin-like proteins have now been characterized from various plant species [193-201]. Genes encoding acidic and basic forms of tobacco PR-5 proteins have been isolated [202, 203] and do not contain introns. At least two domains of the promoter are involved in the induction of acidic PR-5 upon TMV infection [204]. For basic PR-5 a study of tissue-specific expression indicates high expression in roots and stems and a possible involvement of abscisic acid in promoter activation during the response to salt stress. cDNAs encoding PRs-5 from several other plant species have been cloned [54, 197, 205–208].

Concerning the search for a possible biological activity of proteins of the PR-5 group, the first attempts were based on sequence similarity to a bifunctional inhibitor of maize, an inhibitor of both trypsin and insect α-amylase [193]. We did not find this bifunctional activity in any of the isolated tobacco proteins of table V, and it is noteworthy that this activity has also not be reported for any of the numerous thaumatin-like proteins isolated by others (see table VI), including the maize protein zeamatin [199, 209] that was found highly similar, if not identical, to the maize bifunctional inhibitor (Selitrennikoff, personal communication). Transgenic plants expressing constitutively acidic PR-5 were not protected from eating by *Spodoptera exiga* and *H virescens* larvae, as well as not being more resistant to infection by TMV [113].

The first report on biological activity of a PR-5 protein was the demonstration that zeamatin, a protein from maize, was antifungal with membrane permeabilizing activity [210]. Other cereals apparently contained related proteins with similar antifungal activity [199]. Then another group found that tobacco osmotin and the tomato analog was active against *Phytophthora infestans*, responsible for late blight disease of potato [200]. We confirmed and extended these results by showing that several members of the PR-5 group had differential antifungal activities with

specificity for different fungal species [209]. There are now many lines of evidence that proteins of the PR-5 group from various plant species have direct antifungal activity against several classes of fungi (table VI). They have been shown to inhibit hyphal growth and mediate hyphal and spore lysis in *in vitro* assays [197, 199, 200, 209, 210]. It has been proposed that the antifungal activity of the maize PR-5, zeamatin, is the result of permeabilization of fungal membranes [210] through a mechanism that is still operative at 4°C. This capacity may be related to formation of amphipathic domains resulting from secondary structure mediated by disulfide linkages analogous to another type of plant defense proteins, thionins [211]. These amphipathic domains interact with membranes resulting in pore or channel formation, and in extreme conditions membrane lysis.

More information has to be obtained on the molecular mechanisms of antifungal activity of PRs-5. If the effects on fungal membrane permeabilization were confirmed, then a combination of an hydrolytic enzyme of group PR-2 or PR-3 and a PR-5 protein expressed constitutively by genetic engineering would be very likely to confer a high level of protection against infection by fungi, since the two types of proteins would be acting on two different and essential targets of the pathogen.

Other tobacco PR-proteins

Several tobacco PRs have been characterized recently which cannot be classified into the five major groups (table VII).

We have isolated a 45-kDa basic protein [90] which cross-reacts with antisera raised against proteins of groups PR-2, PR-3 and PR-5 (Stintzi, unpublished). The significance of these observations is not understood yet, since the antibodies used have been shown to be very specific to proteins of the corresponding groups in tobacco [67, 91, 92, 181, 186] or in other plant species [97, 135, 212], and no hydrolase activity has been found associated to the purified protein even by using very sensitive enzymatic assays. cDNA cloning is presently in progress.

Another basic protein with a molecular mass of 16 kDa has been found to accumulate in TMV-infected tobacco leaves and its biological activity is also unknown. It does not cross-react with antibodies raised against the acidic PRs-1 (-1a, -1b and -1c) or against the basic counterpart PR-1g (table I). These data and partial microsequencing demonstrate that it does not belong to the PR-1 group in spite of a similar molecular mass. It is presently under investigation.

Two α-amylases are strongly induced in tobacco leaves upon infection by TMV [213]. They are endo-type enzymes and have many features in common with other typical acidic PRs, for instance an apoplastic localization and a high degree of resistance to proteolytic cleavage. Both proteins have an apparent molecular mass of 44-kDa. Like glycanhydrolases of groups PR-2 and PR-3 they can be considered as direct antifungal enzymes since their substrates, 1,4-α-glucans, occur in the cell wall of many fungi.

We have also shown a strong induction of a proteinase inhibitory activity in tobacco during the hypersensitive reaction to TMV [214]. The active inhibitor was purified and shown to be a small polypeptide of the inhibitor I type which displays a strict specificity for serine proteinases of microbial origin, such as subtilisin or proteinase K. Thus, the tobacco inhibitor appears as a good candidate for reducing proteolytic activities of phytopathogenic bacteria or fungi *in planta*. It is noteworthy that the active inhibitors are not monomers, in contrast with most of the other PRs.

Table VII. Other not yet classified tobacco PR-proteins. Proteins TIMPa and TIMPb, amyl 1 and amyl 2, 45 K and 16 K, were isolated and assayed as described in [214, 215], [213], [90], respectively. Proteins P39a and P40a have been isolated recently by the authors (unpublished results) and partial amino acid sequencing has shown identity to a cloned tobacco peroxidase [218]. For all other items, see legend to table I.

Name	*Molecular mass (kDa)*	*Isoelectric point*	*Localization*	*Biological activity*
TIMPa	8	8.7	int	inhibitors of
TIMPb	8	8.7	int	microbial proteases
P39a	39	<7	ext	peroxidase
P40a	40	<7	ext	peroxidase
amyl 1	44	4.5	ext	α-amylase
amyl 2	44	4.2	ext	α-amylase
45K	45	>7	ext	?
16K	16	>7	nd	?

Two closely related cDNAs were cloned recently and shown to encode two active inhibitors whose subunits were separated by HPLC [215]. Southern analysis of genomic DNA. comparison of deduced amino acid sequences and characterization of the two separated proteins (TIMPa and TIMPb, table VII) suggest that the two genes of tobacco are homeologues originating from each parent. We have shown the presence of a glutamic acid residue at the P1 position of the active site, known to determine the specificity of this type of inhibitors. However, the V8 proteinase from *Staphylococcus aureus*, an enzyme that cleaves polypeptides after glutamic residues was not affected by the tobacco inhibitor. We have demonstrated a strong accumulation of both mRNAs and proteins in response to TMV, messengers and products of the two genes being present in a 3/2 ratio in infected leaves as well as in the upper uninfected leaves, the induction being markedly lower, however, at distance from the infection sites. We found that the transcripts also accumulated in sepals and petals of healthy plants, indicating that these genes are developmentally regulated. Unlike the tomato and potato I inhibitors [216], the tobacco inhibitor was only weakly induced by wounding, but was expressed upon treatment with salicylic acid and ethylene, like many other PR-proteins.

We have also isolated two acidic extracellular proteins with peroxidase activity. Peroxidase isozyme induction by TMV infection of tobacco had been studied by others [217] and indicated a preferential induction of two moderately anionic peroxidases called P56 and P61, whereas the anionic isozymes P35 and P37 were already present in uninfected material and only weakly induced during infection. In fact the isoforms P39a and P40a that we have isolated are strongly induced in TMV-infected Samsun NN (Stintzi, unpublished). Surprisingly, partial amino acid sequence analysis indicates identity to P35 whose cDNA has been cloned [218] and which has been described as a lignin-forming enzyme, based on its high expression in the lignified stem and on its affinity for coniferyl alcohol. In our group we are also studying the induction of aromatic metabolism around the virus lesions and already have evidence for an increased incorporation of phenolics into the cell wall [219, 220] that is perhaps catalysed by P39a and P40a. Further studies on these peroxidases are under way to understand their function and role in defense.

Conclusion

This special issue is dedicated to Professor Léon Hirth who initiated part of the work described above and encouraged three of us, P Geoffroy, M Legrand and B Fritig already in the early 1970s to develop a research project on the response of plants to exposure to pathogens, namely his favourite pathogens, viruses. Endowed with an outstanding scientific intuition, he suggested already at that time that the hypersensitive response to viruses would be a particularly good model to study.

In fact the hypersensitive response (HR) of plants to pathogens is one of the most efficient natural mechanisms of induced defense of plants against pathogens. This is also true in the case of virus infections, viruses being the inducers in our model system. In hypersensitivity to viruses the same characteristics are found as in incompatible interactions between plants and fungi or bacteria [9, 10]: i) necrosis at and around each point at which the leaf tissue was infected; ii) localization of the pathogen to the region of each initiated infection; and iii) induction of marked metabolic changes in the cells surrounding the necrotic area, these changes being believed to cause, or at least to contribute to, the resistance observed. Furthermore, the hypersensitive response to viruses involves a cascade of events and signals [18] similar to that proposed for active defense against fungi and bacteria: the response would be initiated by a specific gene-for-gene recognition between the plant and the virus that would lead to cell damage (death of a number of cells) and the release of intermediary signals which in tum would enter a reception-transduction pathway leading to major changes in gene expression (usually gene activation) responsible for the resistance observed.

Under at first the tutelage of Professor Hirth and later his support through stimulating discussions and encouragements, our group has studied particularly the downstream part of this cascade, *ie* the changes in host gene expression and their possible contribution to the defense response. For some time we have invested efforts to understand the role of plant phenolics in disease resistance, and these efforts are still continuing with some focus on the role of cell wall bound phenolics [221]. However, since 1985, major emphasis has been put on a group of strongly induced proteins with typical properties, the PR-proteins also referred to as 'stress proteins'. Tobacco responding hypersensitively to TMV proved to be a particularly useful experimental system, as anticipated by Professor Léon Hirth, and this model is still recognized as the prototype system from which we have characterized biochemically the highest number and widest array of PRs. Among the 33 proteins isolated, 31 have been characterized and 25 fall into five groups of ubiquitous proteins. We have identified the enzymatic activities and functions of proteins of groups PR-2 and PR-3 as the hydrolytic enzymes 1,3-β-glucanases and chitinases-lysozymes, respectively. We have also participated in the discovery that PR-5 proteins had antifungal activity, and presently, are participating in a

collaborative work aiming to study the antifungal properties of proteins of group PR-1, the initially discovered group of PRs [1, 2]. The PR-4 group is still without known biological activity. Other proteins characterized in tobacco are the hydrolases α-amylases, inhibitors of microbial proteases, and peroxidases. With the exception of the latter, all activities characterized appear to be direct antimicrobial activities (antifungal and/or antibacterial). Some of the proteins also exhibit indirect antimicrobial activities, for instance the elicitor-releasing hydrolases and peroxidases that are likely to catalyse cell wall cross-linking of phenolics.

Very surprisingly, none of the characterized proteins appears to have direct antiviral activity, even though all of them are induced in response to the virus and this response obviously includes the induction of an antiviral state that finally localizes the virus. It has been reported that two antiviral proteins called gp35 and gp22 were purified from tobacco and characterized by monoclonal antibodies to human β-interferon [222]. Later it was shown [223] that gp35 corresponded to the basic, vacuolar form of 1,3-β-glucanase (gluc b of table II) and gp22 was related to both the acidic and basic forms of PR-5 from tobacco. We have tested gluc b (table II) and osmotin (table V) using exactly the bioassay of reference [222] and could not find any antiviral activity associated to these proteins. In fact the bioassay itself is not really representative of what happens in hypersensitively-induced resistance to the virus. The mechanism to be unravelled is not an inhibition of establishment of virus infection, but rather a mechanism responsible for inhibition of virus spread. This mechanism might rely on yet undetected component(s), it might also result from the wide range and superposition of metabolic alterations, for instance the cell wall thickening that could affect the viral 'movement' proteins(s).

As described above, the prospects for genetically-engineered plants with improved resistance against fungi and bacteria by transformation with PR genes are promising, especially if combinations of PRs are expressed with potential synergistic effects or with different target components in the microbe. This still does mimic only partially natural defense which involves a wide array of direct and indirect antimicrobial compounds. The natural defense system could be better approached by manipulating the signals that trigger a battery of defense components, and the characterization of such signals and their manipulation is now under study in many laboratories all over the world.

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