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Review

The role of plant defence proteins in fungal pathogenesis

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SUMMARY

It is becoming increasingly evident that a plant–pathogen interaction may be compared to an open warfare, whose major weapons are proteins synthesized by both organisms. These weapons were gradually developed in what must have been a multimillion-year evolutionary game of ping-pong. The outcome of each battle results in the establishment of resistance or pathogenesis. The plethora of resistance mechanisms exhibited by plants may be grouped into constitutive and inducible, and range from morphological to structural and chemical defences. Most of these mechanisms are defensive, exhibiting a passive role, but some are highly active against pathogens, using as major targets the fungal cell wall, the plasma membrane or intracellular targets. A considerable overlap exists between pathogenesis-related (PR) proteins and antifungal proteins. However, many of the now considered 17 families of PR proteins do not present any known role as antipathogen activity, whereas among the 13 classes of antifungal proteins, most are not PR proteins. Discovery of novel antifungal proteins and peptides continues at a rapid pace. In their long coevolution with plants, phytopathogens have evolved ways to avoid or circumvent the plant defence weaponry. These include protection of fungal structures from plant defence reactions, inhibition of elicitor-induced plant defence responses and suppression of plant defences. A detailed understanding of the molecular events that take place during a plant–pathogen interaction is an essential goal for disease control in the future.

INTRODUCTION

In their long association with pathogens, plants evolved an intricate and elaborate array of defensive tools. At the same time, those very same pathogens developed means to overcome plant resistance mechanisms in what must have been a multimillion-year evolutionary game of ping-pong (Keen, 1999b). As each defensive innovation was established in the host, new ways to circumvent it evolved in the pathogen. Over time, the coevolutionary struggles between would-be pathogens and their erstwhile hosts have generated some of the most complex and interesting interactions known to biology (Taylor, 1998). A plant–pathogen interaction may therefore be considered as an open warfare, whose major weapons are proteins synthesized by both organisms (Ferreira *et al.*, 2006).

The cuticle and the plant cell wall, for example, are pre-formed physical barriers often claimed to constitute the first line of plant defence by protecting against pathogen penetration. In addition, they are also a source of signals used by the invading pathogens to activate their responses or by plants to induce defence mechanisms. Nevertheless, recent evidence challenged the traditional view of the plant cell wall as a passive structural barrier to pathogen invasion. Apparently, plants are able to sense perturbation of the cell wall by monitoring the integrity of its structure. For example, mutations in the Arabidopsis cellulose synthase gene *CESA3* exhibit constitutive activation of jasmonate- and ethylene-mediated defence gene expression and enhanced resistance to powdery mildew pathogens (Cano-Delgado *et al.*, 2003; Nishimura *et al.*, 2003). The papilla is a local cell-wall fortification formed on the inner side of the plant cell walls at the site of pathogen penetration that is regarded as an inducible structural barrier. In the case of powdery mildews, papillae have been reported to play an important role against fungal invasion (Thordal-Christensen *et al.*, 2000; Zeyen *et al.*, 2002).

Fungal pathogens almost invariably trigger cell wall-associated defence responses, such as extracellular hydrogen peroxide generation and callose deposition, when they attempt to penetrate either resistant or susceptible plant cells. Expression of these defences involves communication between the plant cell wall and the cytosol across the plasma membrane. Indeed, peptides containing an Arg–Gly–Asp (RGD) motif which interfere with

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plasma membrane–cell wall adhesion or disruption of plant microfilaments reduce the expression of cell wall-associated defence responses during the penetration of non-host plants by biotrophic fungal pathogens with the consequent increment in fungal penetration efficiency (Mellersh and Heath, 2001).

The actin cytoskeleton has been shown to contribute to both pre-invasion resistance and papillary callose formation in the interactions between *Arabidopsis thaliana* and non-adapted *Colletotrichum* species (Shimada *et al.*, 2006). This study showed an extensive reorganization of actin microfilaments leading to polar orientation of large actin bundles towards appressorial contact sites. Non-adapted *Colletotrichum* species differentiate melanized appressoria on Arabidopsis leaves but fail to form intracellular hyphae. Analyses of non-pathogenic *C. lagenarium* mutants indicated penetration-peg formation as the inductive cue for papillary callose formation. However, the incidence of papilla formation at fungal entry sites is greatly reduced during the compatible interaction of Arabidopsis with *C. higginsianum*, indicating that this adapted pathogen may suppress pre-invasion resistance at the cell periphery (Shimada *et al.*, 2006). Indeed, biotrophic fungi may manipulate the plant cell wall surveillance system for the establishment of biotrophy, subverting the interconnected plant defence signalling pathways or the underlying resistance mechanisms (Jones and Takemoto, 2004; Schulze-Lefert, 2004).

Recent observations have revealed that molecular processes occurring at and in plant cell walls may also function in fungal pathogenesis. The β-1,3-D-glucan callose is rapidly synthesized and deposited at plant cell wall upon microbial attack. Arabidopsis mutants in the gene encoding the single glucan synthase responsible for papillary callose synthesis exhibit broad-spectrum enhanced resistance to powdery mildew fungi, suggesting a role for the wild-type gene in fungal colonization of the host cells. Callose may facilitate penetration of pathogens into host cells by providing a structural collar for the intruder. It has been suggested that the glucan synthase participates in the containment of pathogen-derived elicitors at infection sites, thereby preventing their perception by the plant, or in the protection of the invading pathogen against plant-derived antimicrobial compounds (Gomez-Gomez and Boller, 2002; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003).

Therefore, emerging evidence suggests that pathogens may take over selected aspects of plant gene expression to their own benefit. In this way, they may induce the expression of some components required for the infection or development processes, or they may repress components of the host defence system, such as, for example, proteins of the cytoskeleton. In *Arabidopsis thaliana* and barley (*Hordeum vulgare*), for example, the presence of specific isoforms of the family of heptahelical plasma membrane-localized MLO proteins is required for successful host-cell invasion by powdery mildew fungi (Panstruga, 2005). This study concluded that powdery mildew fungi appear specifically to corrupt MLO to modulate vesicle-associated processes at the plant cell periphery for successful pathogenesis.

TARGETS FOR THE PLANT DEFENCE MECHANISMS

The fungal cell wall

The molecular model of the cell wall of *Saccharomyces cerevisiae* that is generally accepted (Klis *et al.*, 2006; Theis and Stahl, 2004) contains three major classes of carbohydrate polymers, chitin, β-1,3-glucan and β-1,6-glucan, and two main classes of glycosylated cell wall proteins (CWPs). There is no complete model of the cell wall of filamentous fungi available yet, but many similarities are expected to occur between the cell walls of these groups of fungi.

Chitin, a natural homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine residues, is the major cell wall component in filamentous fungi (BeMiller, 1965). The concentration of chitin in the cell walls of these organisms (~10%) is significantly higher than in yeasts (2%) (Theis and Stahl, 2004). In Oomycetes, chitin is only a minor component of their cell walls (Barkai-Golan *et al.*, 1978; Schoffelmeer *et al.*, 1999). Therefore, it is not surprising that plants synthesize a large number of defence proteins capable of binding to chitin and chitin oligosaccharides. Most but not all of these proteins bind to chitin through a conserved amino acid sequence known as the chitin-binding domain (Chrispeels and Raikhel, 1991). Many chitin-binding proteins have been isolated from numerous plant species, especially from their seeds. Examples are class I chitinases (Gomes *et al.*, 1996; Leah *et al.*, 1991), lectins such as wheat (*Triticum aestivum*) germ agglutinin (WGA) (Broekaert *et al.*, 1987; Wright *et al.*, 1991), and antimicrobial peptides such as hevein (Broekaert *et al.*, 1992).

One in six aminosaccharide chitin residues can be devoid of an acetyl group (Blackwell, 1988). Deacetylation is a common process involved in chitin–protein interaction and leads to chitosan formation (Blackwell, 1988), another structural polysaccharide found in fungal cell walls. Plant antifungal proteins have been described exhibiting chitosanase activity.

Glucans are the second major component of the fungal cell wall. Apart from β-1,3-glucan, which is the primary glucan, several other linkages such as α-1,3, α-1,4, β-1,4 and β-1,6 have also been detected in fungal cell walls.

The fungal membrane

The fungal plasma membrane is the target for the largest group of antifungal and antimicrobial proteins (Theis and Stahl, 2004). Over 500 naturally occurring proteins have been reported that are believed to interact with the fungal membrane, leading to pore formation, efflux of cellular components and changes in the

membrane potential (Tossi *et al.*, 2000). They exhibit an enormous diversity of structures but share at least two common features (Tossi *et al.*, 2000): a positive net charge under physiological conditions, which promotes interaction with negatively charged microbial surfaces; and an amphipathic structure (i.e. a structure with two faces, one being positively charged and the other neutral or hydrophobic) that allows incorporation into pathogen membranes. A striking difference between higher eukaryotes and fungal membranes concerns the embedded sterols. The plasma membrane of higher eukaryotes contains cholesterol, sitosterol and/or campesterol, whereas fungal membrane ergosterol comprises ~2% of the fungal dry weight (Brennan *et al.*, 1974; Rattray *et al.*, 1975). It is noteworthy to mention that ergosterol is a non-specific fungal elicitor that induces expression of a specific set of plant defence-related genes (Lochman and Mikes, 2006).

Intracellular targets

To enter fungal cells, plant antifungal proteins must pass through the fungal cell wall and membrane. This may explain the low number of reports on plant defence proteins interacting with the plethora of potential fungal intracellular targets.

PREFORMED VERSUS INDUCIBLE DEFENCE MECHANISMS

Plants have evolved a network of intricate and elaborate defence systems that mediate their interaction with the environment. In what concerns the challenge by fungal pathogens, their resistance is determined by an impressive combination of both constitutive and inducible defence mechanisms that involve a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack (Dixon and Harrison, 1990). For example, the completion of the Arabidopsis genome sequence showed that this plant species has a few hundred open reading frames that encode potential surveillance proteins (Dangl and Jones, 2001).

Preformed or passive defences are the first obstacle an invading pathogen has to overcome before disease is established. The typical preformed, constitutive defences are morphological, structural and chemical barriers. A recognized example of a morphological barrier is the height of lips of stomatal guard cells (Keen, 1999a). As shown by Hoch *et al.* (1987), certain fungal rust pathogens possess exquisite detection mechanisms that sense the height of stomatal guard cell lips encountered on susceptible plants. Thus, when the hyphae find a lip of the proper height, they are programmed to undergo a developmental process resulting in the formation of invasive structures that enter the stomata and begin colonization of the leaf interior.

Structural barriers, such as waxes, cutin, suberin, lignin, cellulose, callose and cell wall proteins, are often rapidly reinforced upon the infection process. In addition, plants constitutively produce a plethora of secondary metabolites and antifungal proteins, many of which can act as antimicrobial compounds during defence against microorganisms. These include phenolics of varying structural sophistication, saponins, terpenoids and steroids. Some preformed compounds are directly toxic, whereas others occur as conjugates such as glycosides that are not toxic *per se*, but become poisonous following disruption of the conjugate. Plant glycosides, for instance, are often hydrolysed by vacuolar glycosidases following pathogen invasion, releasing aglycones that may be quite toxic, not only to the invader but also to neighbouring plant cells (Keen, 1999a). By contrast, some plant preformed compounds are toxic as glycosides, but lose toxicity when deglycosylated. The growth of the wheat root-infecting fungus *Gaeumannomyces graminis* var. *tritici* is inhibited by avenacin. Therefore, oat (*Avena sativa*) plants producing this preformed triterpene saponin glycoside exhibit resistance to the pathogen. However, the related oat root pathogen *G. graminis* var. *avenae* produces a glycosidase that removes the sugar residue from avenacin, effectively detoxifying it. Therefore, this strain is not inhibited by avenacin and oat plants are susceptible to it (Osbourn *et al.*, 1994). A mutation in the glycosidase gene rendered *G. graminis* var. *avenae* sensitive to avenacin and incapable of infecting oat plants (Bowyer *et al.*, 1995). By contrast, engineered oat plants that lack or have only trace amounts of avenacin are compromised for disease resistance against the non-host fungal pathogens *G. graminis* var. *tritici* and *Fusarium culmorum* (Papadopoulou *et al.*, 1999).

Besides constitutive defences, an important plant strategy is to initiate defences in response to pathogen attack (Karban and Baldwin, 1997). Inducible resistance mechanisms are active, energy-requiring systems typified by specific recognition of an invader that ultimately leads to the production of proteins or metabolites that are antagonistic to the invader (Keen, 1999a). They are considered the second obstacle an invading pathogen has to face when attempting infection. Inducible defences may have several advantages such as reducing biosynthetic costs of defence or avoiding the fact that other organisms may exploit the defence to their own benefit (Cipollini *et al.*, 2003; Karban and Baldwin, 1997; Zangerl, 2003). In addition, the variation in plant genotype that is caused by inducible defences may reduce the chances that attackers adapt to plant defences (Agrawal and Karban, 1999). Inducible or active defence mechanisms mainly involve the oxidative burst, localized cell death, accumulation of phytoalexins, synthesis of pathogenesis-related (PR) proteins and cell wall strengthening proteins such as hydroxyproline-rich glycoproteins, and enhanced transcription of genes encoding enzymes involved in the flow of carbon from the primary to the secondary metabolism of plants, such as peroxidases, lipooxygenases, superoxide dismutases and phenylalanine ammonia lyase (PAL), a key enzyme in the biosynthesis of phenolic compounds with antimicrobial activity (Montesinos, 2000).

Besides chemical, structural inductive mechanisms are mostly related to the plant cell wall. Modification of the plant cell wall was recognized as a potential resistance mechanism more than 80 years ago (Young, 1926). Lignification and other chemical modifications of plant cells around the sites of infection lead to wall thickening and the formation of local additions or callosities in the paramural space (i.e. the space between the cell wall and the plasma membrane). Nevertheless, formation of the cell wall apposition (CWAs) or papillae is usually accompanied by the induction of co-localized chemical responses (Bestwick *et al.*, 1998; Matern *et al.*, 1995; McLusky *et al.*, 1999; Nicholson and Hammerschmidt, 1992; Schmelzer, 2002; Thordal-Christensen *et al.*, 1997).

Plant cytoskeleton also plays a significant role in inducible disease resistance. Plant actin microfilaments have been implicated in defence against fungal penetration and their disruption leads to the loss of non-host resistance against several non-host pathogens (Kobayashi *et al.*, 1992). Treatment of several non-host plants (e.g. barley and wheat) with cytochalasins, specific inhibitors of actin polymerization, allows several non-host fungi to penetrate the cells of these plants (Kobayashi *et al.*, 1997).

The inducible responses are turned on systematically in the plant in response to attempted infection. First, in the localized response, a spatially confined necrosis is frequently induced that results either from cell death caused by the action of the pathogen in a compatible interaction or from an endogenous plant cell death response following recognition of the pathogen by the host in an incompatible interaction—the hypersensitive response (HR) (Maleck and Dietrich, 1999). The HR is a pathogen-induced, rapid and localized cell suicide at the spot of infection in which the plant cells react to the invading pathogen by a kind of programmed cell death consisting of electrolyte leakage from the cytoplasm and oxidative burst (Montesinos, 2000). As a result, the pathogen remains confined to necrotic lesions near the site of infection. Thus, as described by Chester (1933), HR is a type of blocking necrosis often developed by non-host plants against many plant pathogens that invade their tissues. The knowledge on signal transduction in the HR is still rather incomplete, but several interesting genes have already been identified, including protein kinases and phosphatases, calmodulin genes and others of unknown biochemical function that ultimately activate transcription of defence response genes. Localized acquired resistance in a ring of cells surrounding necrotic lesions ensure that they become fully refractory to subsequent infection (Bonas and Lahaye, 2002; Fritig *et al.*, 1998). Experiments performed in Tetep, a rice (*Oryza sativa*) cultivar resistant to both *Cochliobolus miyabeanus* and *Magnaporthe grisea*, showed that inoculation with either pathogen triggered the HR. However, in rice cv. Nakdong, susceptible to both fungi, *M. grisea* did not cause HR whereas *C. miyabeanus* caused rapid cell death (Ahn *et al.*, 2005).

Within a few hours of the localized necrosis, the plant begins to express a set of defence genes both locally, at the point of infection, and systemically, throughout the rest of the plant (Antoniw *et al.*, 1980a; van Loon, 1985). Thus, local HR often triggers a systemic signal that transduces non-specific resistance throughout the plant, leading to systemic acquired resistance (SAR), which confers long-lasting, enhanced resistance against subsequent infections by a broad spectrum of pathogens (Durrant and Dong, 2004; Ryals *et al.*, 1996; Somssich, 2003; Sticher *et al.*, 1997). In contrast to HR, the development of SAR is slow and gradual (Scheel, 1998). In this systemic defence, the signal spreads from the place of plant–pathogen interaction and is mediated by an interacting set of endogenous defence signalling molecules that have been identified as messengers in plants, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), nitric oxide (NO) or reactive oxygen species (ROS) (Baker *et al.*, 1997; Beckers and Spoel, 2006; Montesano *et al.*, 2003). These messengers interact with specific binding proteins, which are involved in the transcriptional activation of pathogen-responsive genes. These SAR genes are thought to be responsible for the increased resistance of the non-infected, secondary plant tissues to subsequent infections by the same or even unrelated pathogens (Maleck and Dietrich, 1999; Montesano *et al.*, 2003).

Increasing evidence suggests the existence of cross-talk among the induced defence mechanisms (Beckers and Spoel, 2006). Apparently, these are not controlled by independent linear signalling cascades, but components of one pathway may affect the signalling through other pathways (Maleck and Dietrich, 1999). Several examples have been reported on antagonistic or synergistic interactions between defence responses. Current knowledge suggests that plants do not activate a stereotypic defence response against all pathogen attacks, but rather appear to recognize a multitude of components from a particular pathogen to fine-tune a specific response. Experiments performed under laboratory conditions have shown that elicitors, avirulence factors and mechanical stress each only induce a subset of the plant response to pathogens (Gus-Mayer *et al.*, 1998).

PLANT DEFENSIVE WEAPONARY AGAINST FUNGAL PATHOGENESIS

A plant–pathogen interaction may be regarded as an open warfare, whose weapons are proteins and low-molecular-mass compounds synthesized by both organisms. The outcome of each battle results in the establishment of resistance or pathogenesis. This is readily illustrated by the following example.

Plant cell walls, essentially composed of polysaccharides and proteins, are attacked by a range of degrading enzymes liberated by many pathogenic fungi. These hydrolases fragment the plant cell wall polymers releasing oligosaccharides and facilitating colonization of the host cells. The oligosaccharides not only

provide the fungus with a carbon source but are also perceived by and elicit the plant defensive mechanisms. Thus, for example, pectin, a major component of the cell walls in many plants, is cleaved by fungal endopolygalacturonases (EPGs) with the transient formation of elicitor-active oligogalacturonides (OGAs) with degrees of polymerization between 9 and 15. For this reason, OGAs are rapidly converted to smaller, biologically inactive fragments by the EPGs.

To increase the lifetime of the biologically active oligosaccharides, plants release inhibitors of fungal glycanases. These include inhibitors of pectin-degrading enzymes such as polygalacturonases, pectin methyl esterases and pectin lyases, and cross-linking glycan (known earlier as hemicelluloses)-degrading enzymes such as endoxylanases and xyloglucan endoglucanases (Juge, 2006). For example, plant polygalacturonase-inhibiting proteins (PGIPs) are glycoproteins present in the apoplast of many plants that form reversible high-affinity complexes with fungal EPGs, reducing their catalytic activity by one or two orders of magnitude. By limiting EPG activity, the lifetime and concentration of OGAs are increased, prolonging or enhancing plant defence responses (Desiderio *et al.*, 1997; Powell *et al.*, 1995).

Conversely, fragmentation of the fungal cell wall by plant-derived chitinases and β-1,3-glucanases also generates oligosaccharides that induce plant defence responses. In return, fungi produce glucanase inhibitor proteins (GIPs) that prevent degradation of their own cell wall, thus limiting their perception by the plants (Albersheim and Valent, 1974). Major groups of proteins involved in plant defence are the pathogenesis-related proteins and the antifungal proteins, both of which are treated in the next sections of this article. However, plants also produce lower molecular mass defensive compounds. Selected examples, considered below, are ROS and phytoalexins.

ROS, such as superoxide anions, hydroxyl radicals and hydrogen peroxide, play an important role in plant defence during plant–pathogen interactions (Wu *et al.*, 1997). ROS are directly toxic to microbial invaders, catalyse oxidative cross-linking of the cell wall at the site of attempted infection and participate in signalling the onset of other defence responses (Nurnberger *et al.*, 2004). Thus, hydrogen peroxide is produced by plant cells in response to infection (Baker and Orlandi, 1995), triggering the HR (Tenhaken *et al.*, 1995), strengthening cell walls (Brisson *et al.*, 1994) and enhancing lignin formation (Wu *et al.*, 1995). In addition, H_2O_2 directly inhibits pathogen growth (Wu *et al.*, 1995) and induces the synthesis of PR proteins, phytoalexins, SA and ethylene (Chamnongpol *et al.*, 1998; Mehdy, 1994; Wu *et al.*, 1997).

Phytoalexins are low-molecular-mass secondary metabolites of a non-proteinaceous nature which are produced by a broad range of plant species. They display an enormous chemical diversity, exhibit antimicrobial and antifungal activities, are induced by pathogen infection and elicitors (Grayer and Kokubun, 2001; Hammerschmidt, 1999), and are synthesized through complex biochemical pathways such as the shikimic acid pathway (Dixon *et al.*, 1996).

As for chitinases and glucanases, it has been difficult to demonstrate a direct role for phytoalexins in plant resistance to pathogen attack (Punja, 2001). Wild-type *Arabidopsis thaliana* is resistant to *Alternaria brassicicola*, exhibiting a typical HR in response to inoculation with this fungus (Thomma *et al.*, 1998). *pad3-1*, an Arabidopsis phytoalexin-deficient (pad) mutant, is compromised for non-host resistance against *A. brassicicola* (Thomma *et al.*, 1999). *pad3-1* is required for the biosynthesis of the phytoalexin camalexin and encodes a putative cytochrome P450 monooxygenase (Zhou *et al.*, 1999).

Phytoalexins from *Vitis* species, for example, belong to the stilbene family of phenolic compounds and derive from *trans*-resveratrol (3,5,4′-trihydroxy stilbene) (Jeandet *et al.*, 2002). *Trans*-resveratrol has been shown to be excreted from grapevine cell cultures. Among the viniferins, considered as oligomers of resveratrol, ε-viniferin (a cyclic resveratrol dehydrodimer) and α-viniferin (a cyclic resveratrol dehydrotrimer), have been reported to accumulate in grapevine in response to pathogen attack or stress (Jeandet *et al.*, 1997; Langcake and Pryce, 1977; Pryce and Langcake, 1977). ε-Viniferin, for example, increases intracellularly in response to endopolygalacturonase I (elicitor) from *Botrytis cinerea* and to UV-light irradiation. Other stilbenes detected in grapevine include *trans*-pterostilbene, a dimethylated resveratrol derivative (3,5-dimethoxy-4′-hydroxystilbene), *trans*- and *cis*-piceid, a 3-*O*-β-D-glucoside of resveratrol, *trans*- and *cis*-astringin, a 3-*O*-β-D-glucoside of 3′-hydroxyresveratrol, and *trans*- and *cis*-resveratroloside, a 4′-*O*-β-D-glucoside of resveratrol (Jeandet *et al.*, 2002).

PATHOGENESIS-RELATED PROTEINS

The concept of pathogenesis-related protein (abbreviation: PR) was introduced in 1980 to designate any protein coded for by the host plant but induced only in pathological or related situations (Antoniw *et al.*, 1980b), including viral, fungal or bacterial infections, parasitic attack by nematodes, phytophagous insects and other higher forms of animals such as herbivores. Abiotic stresses and disorders were not considered inducers of PR proteins, although certain non-infectious physiological conditions (e.g. toxin-induced chlorosis or necrosis) often trigger induction of certain PR proteins (Jayaraj *et al.*, 2004). More recent reports have shown the induction of PR proteins as a result of colonization by non-pathogenic/beneficial fungi and bacteria (Blilou *et al.*, 2000; Coventry and Dubery, 2001; Yedidia *et al.*, 2000; Zehnder *et al.*, 2001). The major criterion for inclusion among the PR is that the protein (or protein isoform) concerned is newly expressed upon infection, although not necessarily in all pathological conditions (van Loon, 1999). According to this definition, proteins that are constitutively present in low but detectable amounts in healthy tissues but which

are induced under pathological conditions are not considered PR proteins. These concepts were initially based on experiments performed in the 1970s on tobacco (*Nicotiana tabacum*) leaves reacting hypersensitively to tobacco mosaic virus (van Loon and van Kammen, 1970).

PR proteins were initially found to be typically acidic, of low molecular mass, highly resistant to proteolytic degradation and to low pH values, and localized predominantly extracellularly in the intercellular space of leaves. Following infection, they accumulate in leaves and other organs, where they may comprise more than 10% of the total soluble protein.

The term PR-like protein was proposed to accommodate proteins that are present in healthy plants, being induced essentially in a developmentally controlled, tissue-specific manner. These proteins, which are not synthesized in response to pathogen infection or related stresses, are predominantly basic and localized intracellularly in the vacuole (van Loon *et al*., 1994). As new proteins are discovered, a clear distinction between PR and PR-like proteins is sometimes difficult to establish. This is the case, for example, of basic chitinase and glucanase from mature leaves, which can be expressed in a developmentally controlled manner or induced in response to infection in the same organs; and also for acidic and basic glucanase and chitinase, which may be expressed constitutively in floral organs and inducible by pathogens in leaves (van Loon, 1999).

The distinction between PR proteins and PR-like proteins became blurred by the discovery of specific PR proteins in healthy tissues and the induction of PR-like proteins upon pathogen attack. Contrary to the initial definition of PR protein, many authors subsequently considered as PR proteins many proteins whose synthesis is induced in response to biotic stress. Recently, van Loon *et al*. (2006) introduced the general term 'inducible defence-related proteins' to include proteins that are mostly non-detectable in healthy tissues and for which induction at the protein level has been demonstrated after pathogen infection. Therefore, this general term encompasses both known PR protein families and non-classified proteins meeting the above criteria but fails to include many proteins that are present in healthy tissues and are induced upon microbial infection.

The induction of some PR proteins under pathological conditions suggests, but does not prove, a role for these proteins in plant defence (van Loon, 1990). Therefore, these proteins have been generally considered as defence proteins, functioning in preventing or limiting pathogen invasion and spread. However, their contribution to resistance against the initial infection is usually poor. Nevertheless, if they are already present in a tissue, or if they have been induced in non-infected, distant tissues as a result of primary infection in the vicinity, then they confer an enhanced level of protection.

PR proteins are produced by plants during normal development or as part of an induced defence from fungal pathogens, against which they exert biocontrol. Therefore, their biosynthesis and accumulation is considered a major defence mechanism of plants against fungal pathogens (Odjakova and Hadjiivanova, 2001; Somssich and Hahlbrock, 1998). Not only do some of these proteins exhibit antifungal properties *in vitro,* but also they have been shown to be induced *in vivo* in a very large number of plants in response to fungal attack. A large group of PR proteins has been shown to be rapidly and massively induced both locally around infection sites and systemically (Kombrink and Somssich, 1997). PR proteins are also induced in response to various environmental stress factors, such as drought, salinity, wounding, heavy metals, endogenous and exogenous elicitor treatment, and plant growth regulators (Derckel *et al*., 1996; Xie *et al*., 1999; Yu *et al*., 2001).

The PR proteins encompass several different groups of structurally and functionally unrelated proteins, which have been grouped into protein families according to coding sequence similarities, serological relationships, and/or enzymatic or biological activities, although additional pathogen-induced proteins with potential antipathogenic action are consistently being described (Fritig *et al*., 1998; Somssich and Hahlbrock, 1998). Initially, four protein components were detected in hypersensitive tobacco plants, which were designated I, II, III and IV based on their increasing order of electrophoretic mobility (van Loon and van Kammen, 1970). Subsequently, these proteins were classified into five groups, PR-1 to PR-5. Each of these five classical groups of PR proteins comprised two subclasses: an acidic subclass, usually encountered in the extracellular space, whose members are induced by salicylic acid, and a basic subclass, found in the plant cell vacuole, whose members are induced by ethylene or jasmonic acid (Boller *et al*., 1983; Hamel and Bellemare, 1995; Samac *et al*., 1990; Selitrennikoff, 2001; Thomma *et al*., 1998). Seventeen classes are now considered, numbered in the order in which they were discovered, from PR-1 to PR-17 (Table 1). The families are numbered and the different members of the same family are assigned letters according to the order in which they were described. The function of many PR proteins remains a mystery. However, members of several of these families were demonstrated to have damaging actions on the structures of the parasite, thus exhibiting antifungal activity, in *in vitro* bioassays and supporting a possible role for these proteins in plant defence (Kombrink and Somssich, 1997; Odjakova and Hadjiivanova, 2001). These include PR-1 and PR-5 (thaumatin-like proteins and osmotins), which are thought to create transmembrane pores and have therefore been termed permatins; PR-2 (β-1,3-glucanases) and PR-3, 4, 8 and 11 (chitinases), which attack β-1,3-glucans and chitin, respectively, components of the cell walls in most higher fungi (Honée, 1999). In most cases, an assortment of PR proteins belonging to diverse subclasses are induced, rather than a single member of a single family of PR proteins (Datta *et al*., 1999). It is also common for some PR proteins to display synergism. Because chitin and β-1,3-glucan are synthesized

Table 1 Families of pathogenesis-related proteins.

Family	Type member	Biochemical properties	Molecular mass range (kDa)
PR-1	Tobacco PR-1a	Unknown	15–17
PR-2	Tobacco PR-2	β-1,3-glucanase	30–41
PR-3	Tobacco P,Q	Chitinase class I, II, IV, VI, VII	35–46
PR-4	Tobacco R	Chitin-binding proteins	13–14
PR-5	Tobacco S	Thaumatin-like	16–26
PR-6	Tomato inhibitor I	Proteinase inhibitor	8–22
PR-7	Tomato P_{69}	Endoproteinase	69
PR-8	Cucumber chitinase	Chitinase class III	30–35
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase (POC)	50–70
PR-10	Parsley 'PR-1'	'Ribonuclease-like'	18–19
PR-11	Tobacco class V chitinase	Chitinase class V	40
PR-12	Radish Rs-AFP3	Defensins	5
PR-13	*Arabidopsis* THI-2.1	Thionins	5–7
PR-14	Barley LTP4	Lipid transfer proteins	9
PR-15	Barley OxOa (germin)	Oxalate oxidases	22–25
PR-16	Barley OxOLP	Oxalate oxidase-like protein	100 (hexamer)
PR-17	Tobacco PRp27	Unknown	?

Source: modified from van Loon *et al.* (2006).

simultaneously in the apex of growing hyphae of filamentous fungi, the effectiveness of a hydrolase may depend on the simultaneous action of another one to hydrolyse mixed chitin–glucan fibres (Stintzi *et al.*, 1993). Thus, for example, class II β-1,3-glucanases only show antifungal activity *in vitro* when they are applied in combination with chitinases or class I β-1,3-glucanases (Theis and Stahl, 2004). For these reasons, a genetic engineering strategy involving constitutive, high-level expression of combinations of PR proteins with different modes of action against target organisms may provide broad-spectrum, durable resistance to a variety of diseases and pests. Several reports have demonstrated that transgenic plants over-expressing constitutively some PR genes show enhanced resistance to fungal pathogens. Examples include over-expression of chitinases, glucanases and ribosome-inactivating proteins (RIPs) (Alexander *et al.*, 1993; Hong and Hwang, 2006; Jach *et al.*, 1995; Mauch *et al.*, 1988), osmotin (Liu *et al.*, 1994) and others. This strategy ensures that PR proteins are present in the host plant at levels required for effective resistance before the pathogen attack. For example, transgenic tomato plants expressing only a chitinase transgene or a β-1,3-glucanase transgene were susceptible to *Fusarium oxysporum*, but plants expressing both genes had significantly higher resistance than the plants expressing only one of these two enzymes (Jongedijk *et al.*, 1955).

There is also evidence that at least some members of the other PR protein families display a role against pathogen attack or predation. Thus, members of the PR-1 family have been associated with activity against oomycetes (van Loon *et al.*, 2006). PR-6 proteins (proteinase inhibitors) may target nematodes and herbivorous insects, whereas the PR-7 protein (an endoproteinase) may be involved in microbial cell wall dissolution (Jordá *et al.*, 2000). The peroxidase activity of the PR-9 family may act in cell wall reinforcement by catalysing lignification, leading to enhanced resistance against multiple pathogens (Passardi *et al.*, 2004), whereas some members of the PR-10 family exhibit a weak ribonuclease activity, suggesting a role in defence against viruses (Bufe *et al.*, 1996; Park *et al.*, 2004a). Members of the PR-12 (defensins), PR-13 (thionins) and PR-14 (lipid transfer proteins) families display antibacterial and antifungal activities (Bohlmann, 1994; Epple *et al.*, 1997; Garcia-Olmedo *et al.*, 1995; Lay and Anderson, 2005; Thomma *et al.*, 2002). PR-15 (oxalate oxidases) and PR-16 (oxalate oxidase-like proteins) proteins generate hydrogen peroxide that may be toxic to attackers or stimulate plant defence responses (Bernier and Berna, 2001; Donaldson *et al.*, 2001; Hu *et al.*, 2003). PR-17 proteins, as yet uncharacterized, have been detected in infected tobacco, wheat and barley (Christensen *et al.*, 2002).

Let us consider, for example, the case study of grapevine (*Vitis vinifera*). A considerable number of studies have now been published on the induction of PR proteins in vine plants and on their inevitable accumulation in grapes during the growing season (Tattersall *et al.*, 2001). This may occur in healthy grape berries during the normal fruit development, with véraison (the French term used by viticulturalists to denote the inception of ripening) apparently being the trigger for gene expression, or as a part of an induced defence against the classical PR protein gene inducers, stress and pathogenic attack. Taken together, these processes modulate the levels and proportions of the PR proteins in grapes, in a way that seems to depend on the cultivar, region, climate and agricultural practices (Ferreira *et al.*, 2001, 2004). For these reasons, the precise pattern of PR proteins that accumulate

in mature berries is determined by the precise environmental and pathological conditions that prevail during vegetative growth (Monteiro *et al.*, 2003b).

ANTIFUNGAL PROTEINS

Introduction

Antifungal proteins, as their name implies, serve a protective function against fungal invasion. They are involved in constitutive and induced resistance to fungal attack and are produced by a multitude of organisms including flowering plants, gymnosperms, fungi, bacteria, insects, molluscs and mammals (Ng, 2004; Selitrennikoff, 2001). Plant seeds are especially rich in antimicrobial proteins, with levels that are several fold higher than those present in leaves or flowers (Wang *et al.*, 2001).

A spectacular diversity of amino acid sequences has been reported for antifungal proteins, with hundreds of them already known and with more being discovered almost daily (Ng, 2004; Selitrennikoff, 2001). C. P. Selitrennikoff considered 13 classes of antifungal proteins (see Table 2), which were named primarily on the basis of their mechanisms of action (e.g. chitinases, β-glucanases), their structure (e.g. glycine rich) or their similarity to a known type of protein (e.g. thaumatin-like protein) (Selitrennikoff, 2001; Theis and Stahl, 2004; Wang *et al.*, 2005). Nevertheless, several proteins may be and have been classified into more than one group.

A comparison between Tables 1 and 2 illustrates the correspondence that occurs between the 13 classes of antifungal proteins proposed by C. P. Selitrennikoff (Selitrennikoff, 2001) and the 17 families of PR proteins established by van Loon and colleagues (van Loon *et al.*, 2006). There is partial overlap but not a complete match between the terms antifungal proteins on the one hand and PR proteins or inducible defence-related proteins on the other. However, several of the 17 families of PR proteins do not exhibit any known antifungal activity, whereas many antifungal proteins described in the literature are not PR proteins. The most important difference that emerges between antifungal proteins and PR proteins is that the latter are induced in plants in response to infection (and are not necessarily antimicrobial), whereas the former can be present in any kind of organism (and are not necessarily induced).

It is important to note that there is a substantial variation in the effectiveness of closely related proteins against different fungi, even within individual classes or families of both PR proteins and antifungal proteins. Thus, for example, PR-1 proteins and chitinases

Table 2 Classes of antifungal proteins.

Class	Occurrence	Major characteristics	Mechanism of action
PR-1 proteins	Plants	Molecular masses of 15–17 kDa. Homology to the superfamily of cysteine-rich proteins	Unknown
β-Glucanases	Microorganisms, plants, invertebrates and vertebrates	1,3-β-Endoglucanase activity	Hydrolysis of the structural 1,3-β-glucan present in the fungal cell wall
Chitinases	Viruses, bacteria, fungi, snails, fish, plants, insects, mammals and amphibians	Chitinase activity. Molecular masses of 26–43 kDa	Cleave cell wall chitin polymers *in situ*
Chitin-binding proteins	Bacteria, plants, insects and crustaceans	Molecular masses of 3.1–20 kDa. Chitin-binding proteins	Binding to chitin (?)
Thaumatin-like proteins	Plants	Molecular masses ~22 kDa. Share significant amino acid homology to thaumatin	Not completely understood. Some cause fungal cell permeability changes, others bind to 1,3-β-glucan and exhibit 1,3-β-glucanase activity
Defensins/thionins	Mammals, fungi, insects and plants	Low-molecular-mass, cysteine- rich proteins	Fungal inhibition probably occurs through an ion efflux mechanism
Cyclophilin-like proteins	Bacteria, plants, animals and fungi	Example: mungin	Unknown
Glycine/histidine-rich proteins	Insects	Extremely rich in glycine and histidine, which may comprise as much as 80% of the amino acids	Unknown
Ribosome-inactivating proteins (RIPs)	Fungi and plants	RNA *N*-glycosidases that depurinate rRNA	Inactivate fungal ribosomes *in vitro* and, presumably, *in situ*
Lipid transfer proteins (LTPs)	Mammals, plants, fungi and bacteria	Molecular masses of ~8.7 kDa	Unknown
Killer proteins (killer toxins)	Yeasts	Yeast cells secreting a killer toxin are resistant to their own toxin but are sensitive to other toxins	Varied mechanisms of action
Protease inhibitors	Plants, animals and microorganisms	Protein inhibitors of serine and cysteine proteases	Unknown
Other proteins	Plants	Examples: viridin and snakin-1	Unknown

have been described which are totally devoid of antifungal activity. Moreover, more than a 100-fold difference in anti-oomycete activity has been reported for various PR-1 proteins.

The target structures of the antifungal proteins range from the outermost part of the fungal cell, the cell wall, to the plasma membrane and finally to several intracellular targets (Theis and Stahl, 2004). Therefore, these proteins exhibit a very wide diversity of action mechanisms, including, for example, inhibition of the synthesis of the fungal cell wall or disruption of its structure and/or function, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis and inhibition of the cell cycle. Nevertheless, the mode of action of most of these proteins remains to be elucidated (Ng, 2004; Selitrennikoff, 2001).

PR-1 proteins

The PR-1 family is often the most abundant group of proteins and is induced to very high levels upon infection, reaching up to 1–2% of the total leaf protein (Jayaraj *et al.*, 2004). This family is strongly conserved and has been detected in every plant species examined to date. Homologues have been encountered in fungi, insects and vertebrates, including humans (van Loon and van Strien, 1999).

Although the biological function of PR-1 proteins has not yet been established and their mechanism of action is not understood, plant PR-1 proteins exhibit antifungal activity both *in vitro* and *in planta* (e.g. in transgenic plants over-expressing tobacco PR-1) (Niderman *et al.*, 1995; Tahiri-Alaoui *et al.*, 1993). An association between PR-1 proteins and enhanced resistance against oomycetes has been suggested. However, not enough data have been reported in the available literature to rule out a direct role of PR-1 against non-oomycete pathogens (van Loon *et al.*, 2006). Notably, the prominent PR-1 proteins are often used as markers of the enhanced defensive state conferred by pathogen-induced SAR (van Loon *et al.*, 2006).

PR-1 proteins may be divided into two groups, one being acidic and the other basic (sequence similarity between the two groups is about 65%) (Jayaraj *et al.*, 2004). In tobacco, at least 16 PR-1-type genes were detected (Cornelissen *et al.*, 1987). Three acidic proteins (1a, 1b and 1c) and one basic (1g) protein were found to be induced upon tobacco mosaic virus infection (van Loon *et al.*, 1994). The fully sequenced genomes of Arabidopsis and rice include 22 and 39 PR-1-type genes, respectively (van Loon *et al.*, 2006). In Arabidopsis, a single PR-1 gene is activated upon infection, insect attack or chemical treatment, but ten and eight different PR-1-type genes are constitutively expressed in roots and pollen, respectively (van Loon *et al.*, 2006).

β-Glucanases

Laminarinases (β-1,3-endoglucanases; EC 3.2.1.6) are present in a wide variety of plants (including vegetative parts and seeds), animals (vertebrates and invertebrates) and microorganisms (Jwanny *et al.*, 2001).

Plant β-1,3-glucanases are referred to as PR-2 proteins and are subdivided into three classes. Class I glucanases are basic proteins of about 33 kDa and are localized in the plant vacuole (Bulcke *et al.*, 1989). Classes II and III include acidic, extracellular proteins of about 36 kDa (Theis and Stahl, 2004). They participate in several physiological and developmental plant processes. In addition, class I β-1,3-glucanases exhibit antifungal activity both *in vitro* and *in planta*, using transgenic plants over-expressing a PR-2 protein (Joshi *et al.*, 1998; Mauch *et al.*, 1988). Class II β-1,3-glucanases exhibit *in vitro* antifungal activity only if applied in combination with chitinases or class I β-1,3-glucanases (Theis and Stahl, 2004).

Chitinases

Chitinases (EC 3.2.1.14) constitute the second largest group of antifungal proteins. They catalyse the hydrolytic cleavage of the β-1,4-glycoside bond present in biopolymers of *N*-acetyl-D-glucosamine, mainly in chitin. Chitinases can be grouped in two categories: exochitinases, acting on non-reducing ends of the chitin chain, and endochitinases, which hydrolyse internal bonds (Kasprzewska, 2003). In general, these enzymes catalyse chitin degradation, acting most often as endochitinases and producing chito-oligosaccharides of 2–6 *N*-acetyl-D-glucosamine residues in length (Stintzi *et al.*, 1993).

Chitinases are classified in families 18 and 19 of the 57 families in which *O*-glycoside hydrolases are presently subdivided (Henrissat and Bairoch, 1996). Higher plants synthesize seven different classes of chitinases which differ in protein structure, substrate specificity, mechanism of catalysis and sensitivity to inhibitors (Brunner *et al.*, 1998). For example, unlike classe II chitinases, class I chitinases contain a chitin-binding, hevein-like domain identical to that of chitin binding proteins (Theis and Stahl, 2004). These classes are grouped into three families of PR proteins (Neuhaus *et al.*, 1996; Table 1): chitinases of classes Ia, Ib, II, IV, VI and VII belong to the PR-3 family, whereas those of classes III and V are included in the PR-8 and PR-11 families, respectively. Additionally, some proteins with low endochitinase activity occur in the PR-4 family (chitin-binding proteins) (Melchers *et al.*, 1994). Acidic chitinases belonging to classes Ib, II, III, IV and VI are secreted to the apoplast, whereas basic chitinases included in classes Ia, III and VI are located in vacuoles (Arie *et al.*, 2000).

Chitinases have been found in a very wide range of organisms, containing or not containing chitin, such as viruses, bacteria, fungi, plants (gymnosperms and angiosperms) and animals (insects, snails, fish, amphibians and mammals) (Goormachtig *et al.*, 1998). For example, a 30.8-kDa chitinase with antifungal activity has been isolated from mung bean (*Phaseolus mungo*) seeds (Wang *et al.*,

2005), whereas two 28-kDa chitinases designated chitinase A and chitinase B also exhibiting antifungal activity were characterized in maize (*Zea mays*) seeds (Huynh *et al.*, 1992). Interestingly, some chitinases such as dolichin (28 kDa; Graham and Sticklen, 1994; Ye *et al.*, 2000a), delandin (28 kDa; Ye and Ng, 2002a) and pananotin (35 kDa; Lam and Ng, 2002), present in field bean (*Dolichos lablab*) and ricebean (*Delandia umbellata*) seeds and sanchi ginseng (*Panax notoginseng*) roots, respectively, exhibit antifungal activity and also cell-free translating inhibiting activity and inhibitory activity against HIV-1 reverse transcriptase (Ng, 2004).

Chitinases, as with many other PR proteins, may be synthesized in both a constitutive and an inducible manner. Some chitinase forms, both apoplastic and vacuolar, are synthesized constitutively in healthy plants in a developmentally and tissue- and organ-specific mode. In addition, chitinases, again as with many other PR proteins, are also up-regulated by biotic and abiotic stresses, such as fungal challenge, wounding, drought, cold, ozone, heavy metals, excessive salinity and UV-light, and treatment with phytohormones such as ethylene, jasmonic acid and salicylic acid (Kasprzewska, 2003).

Chitinases are apparently involved in numerous physiological processes, including development and growth. In those organisms that contain chitin, they are presumably required for morphogenesis of cell walls and exoskeleton (Gooday, 1971). For instance, in yeast and various fungi they take part in remodelling cell wall structure and daughter cell separation (Cohen-Kupiec and Chet, 1998; Patil *et al.*, 2000; Shimono *et al.*, 2002). A role in nutrition is fulfilled in some bacteria species that secrete chitinases and are able to grow on chitin as their only carbon source (Wang and Chang, 1997; Wang *et al.*, 2002; Watanabe *et al.*, 1999). Even in plants, chitinases have been reported to play a role in growth and development, during the nodulation process or in programmed cell death (Collinge *et al.*, 1993; Cullimore *et al.*, 2001; De Jong *et al.*, 1992; Goormachtig *et al.*, 1998; Helleboid *et al.*, 2000; van Hengel *et al.*, 1998; van der Holst *et al.*, 2001; Passarinho *et al.*, 2001; Regalado *et al.*, 2000). A chitinase from *Musa* spp. behaves as a fruit-specific vegetative storage protein (Peumans *et al.*, 2002), whereas chitinases from monocotyledonous plants have been reported to display antifreeze activity, suggesting a role in plant frost resistance (Yeh *et al.*, 2000). Chitinases exhibiting aspartic protease inhibitor activity (in *Solanum tuberosum* tubers; Guevara *et al.*, 1999) or α-amylase inhibitor activity (*Coix lochrymajobi*; Ary *et al.*, 1989) have also been reported.

Plant chitinases that hydrolyse chitin inhibit the growth of fungi and generate chitin oligosaccharides that act as elicitors. In addition, many chitinases are induced by pathogen attack and some isoforms exhibit *in vitro* antifungal properties. For these reasons, chitinases are believed to play a major role in plant host defence against pathogens. Nevertheless, their precise role in plant disease resistance has been difficult to establish in non-transgenic plants because chitinases are often present in both resistant and susceptible tissues and their expression is triggered by many inducers other than pathogen attack (Punja and Zhang, 1993). Based on early work on the characterization of plant chitinases made by Thomas Boller and Fred Meins (Neuhaus *et al.*, 1991), Collinge *et al.* (1993) and Kasprzewska (2003) suggested that chitinases fulfil a double function in the protection against fungal colonization. Apparently, apoplastic chitinases function in the early stages of pathogenesis in the signalling process that informs plants about the attack. Indeed, partial digestion of chitin releases oligosaccharides that are perceived by the plant cells as elicitors which, in turn, switch on the active plant defence mechanisms. During the subsequent phases of pathogenesis that follow fungal penetration, vacuolar chitinases released by hyphae-induced, protoplast burst directly repress fungal growth by degrading the newly synthesized chitin chains. This hypothesis is supported by the substrate specificity of cell wall and vacuolar enzymes (Collinge *et al.*, 1993).

It should be noted, however, that many chitinases do not show any antifungal activity *in vitro*. For example, of the two chitinases present in chickpea (*Cicer arietinum*) cell-suspension cultures, only the basic form possessed antifungal activity (Vogelsang and Barz, 1993). In addition, bacterial family 18 chitinases do not have antifungal activity (Theis and Stahl, 2004).

The antifungal activity displayed by many chitinases was initially assumed to derive from their ability to digest chitin, leading to a weakened fungal cell wall and subsequent cell lysis. However, recent evidence indicates that the mechanisms by which chitinases inhibit fungal growth seem to be more dependent on the presence of a chitin-binding domain than on chitinolytic activity. Thus, the antifungal activity of a tobacco class I chitinase is three times higher when a chitin-binding domain is present (Iseli *et al.*, 1993), whereas a mutant class I chitinase from chestnut (*Castanea sativa*) seeds displaying no chitinolytic activity exhibits as much antifungal activity as the wild-type chitinase (Garcia-Casado *et al.*, 1998). By contrast, a mutant class II chitinase from barley showing no chitinolytic activity possesses only 15% of the antifungal activity displayed by the wild-type chitinase (Andersen *et al.*, 1997). Also, a class I chitinase from rye (*Secale cereale*) contains a chitin-binding domain devoid of antifungal activity and a catalytic domain capable of inhibiting fungal growth (Taira *et al.*, 2001).

Chitin and β-1,3-glucan are synthesized simultaneously in the apex of growing hyphae of filamentous fungi (Theis and Stahl, 2004). Therefore, it is not surprising the number of studies that have reported a synergistic action between chitinases and β-1,3-glucanases in the hydrolysis of mixed chitin-glucan fibres, both *in vitro* and *in vivo* (Jach *et al.*, 1995; Stintzi *et al.*, 1993). In fact, the double function proposed by Kasprzewska (2003) for the antifungal activity of chitinases may well be extended to β-1,3-glucanases, with an indirect antifungal activity resulting from partial digestion of chitin and glucans and the corresponding release of elicitors,

and a direct antifungal activity derived from digestion of mixed chitin–glucan fibres and the resulting weakening of fungal cell walls (Ryan and Farmer, 1991).

Chitin-binding proteins

Plant chitin-binding proteins have been classified as PR-4 proteins (Theis and Stahl, 2004; Tables 1 and 2) and are usually subdivided into two classes: class I PR-4 proteins contain a chitin-binding domain similar to a domain present in hevein (a protein from rubber latex; Parijs *et al.*, 1991) and belong to the superfamily of chitin-binding lectins; class II PR-4 proteins lack the chitin-binding hevein domain (Selitrennikoff, 2001; Theis and Stahl, 2004). A chitin-binding protein, inducible by ethylene, has been purified from the leaves of guilder rose (*Hydrangea macrophyla*) (Yang and Gong, 2002).

The antifungal activity of chitin-binding proteins is mainly due to their ability to bind fungal cell wall chitin, which results in disruption of cell polarity and consequent inhibition of growth by mechanisms that have not been elucidated (Bormann *et al.*, 1999). It is possible that the antifungal activity of at least some chitin-binding proteins is just a side-effect (Theis and Stahl, 2004).

Chitin-binding proteins that exhibit antifungal activity but that are not PR-4 proteins have been isolated from a number of sources including bacteria, plants, insects and crustaceans (Selitrennikoff, 2001). In addition, chitin-binding peptides (hevein- and knottin-type, 36–40 residues in length) have been found in several plant seeds (Punja, 2001).

One particular case of chitin-binding proteins that deserves special mention are the vicilins. Indeed, a considerable number of reports indicate that vicilins may be considered as class II chitin-binding proteins, but not class II PR-4 proteins.

The most abundant proteins in legume seeds are the globulins, which comprise the legumins (11S) and the vicilins (7S) and usually account for approximately 80% of the total protein in their mature seeds (Derbyshire *et al.*, 1976). Therefore, vicilins are seed storage proteins of the 7S globulin family, which are present in the seeds of leguminous and other plants (Casey *et al.*, 1986; Derbyshire *et al.*, 1976). They are oligomeric proteins (150–170 kDa) with variable degrees of glycosylation, composed of three similar subunits of ~40–70 kDa, with no disulphide linkages and stabilized by non-covalent forces (Casey *et al.*, 1986; Shutov *et al.*, 1995). The combination of multiple structural genes and extensive post-translation processing (proteolysis and glycosylation) results in a high degree of subunit polymorphism for these proteins (Higgins, 1984). Nevertheless, vicilins from different legume seeds exhibit a considerable amount of sequence homology and have similar three-dimensional protein structures (Argos *et al.*, 1985; Ko *et al.*, 1993; Lawrence *et al.*, 1990, 1994).

Vicilins isolated from the seeds of the legumes cowpea (*Vigna unguiculata*), adzuki bean (*Vigna angularis*), jack bean (*Canavalia ensiformis*), soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and lima bean (*Phaseolus lunatus*) were shown to be immunologically related and to bind strongly to chitin, chitosan and fully acetylated chitin (Firmino *et al.*, 1996; Gomes *et al.*, 1998a; Sales *et al.*, 1996). Association of vicilin to chitin has been shown to be dependent on tryptophan residues in the molecule (Miranda *et al.*, 1998).

Vicilins from different legume seeds have detrimental effects on development of the cowpea weevil (*Callosobruchus maculatus*), a bruchid insect which is a pest of cowpea seeds (Macedo *et al.*, 1993; Yunes *et al.*, 1998). Although the mechanism of action of vicilins upon bruchids is not yet completely understood, these effects have been attributed to the binding of vicilins to the chitinous structures present in the mid-gut of insects (Firmino *et al.*, 1996; Sales *et al.*, 2001). Interestingly, vicilins from all non-host seeds, including those of the *C. maculatus*-resistant cowpea line, strongly inhibit larval development. However, vicilins from *C. maculatus*-susceptible cowpea line and adzuki bean seeds are the exception.

In addition, vicilins from *V. unguiculata* and other legume seeds interfere with the germination of spores or conidia of phytopathogenic fungi and bind to fungal structures, possibly chitin-containing structures of the cell wall (Gomes *et al.*, 1997). For example, *V. unguiculata* vicilins affect growth and inhibit spore germination of the pathogens *Fusarium solani*, *Fusarium oxysporum*, *Collectotricum musae*, *Phytophthora caprici*, *Neurospora crassa* and *Ustilago maydissporidia*, bind to chitin-like structures of *Saccharomyces cerevisiae* and lead to abnormal development (sporulation) of yeast cells (Gomes *et al.*, 1998a,b). Vicilin-related basic proteins isolated from cotton (*Gossypium hirsutum*) seeds have also been shown to inhibit the growth of various filamentous fungi (Chung *et al.*, 1997).

A common property of seed storage proteins is that they are synthesized in high levels in certain developmental stages and accumulate in discrete vesicles called protein storage vacuoles. Therefore, they act as a reserve for surplus organic carbon, nitrogen and sulphur (Pernollet, 1978). Some storage proteins had already been reported to contribute to plant defence mechanisms (Shewry *et al.*, 1995). Nevertheless, it was unexpected to find the vicilins as a group of defensive proteins. Vicilins may therefore be considered multifunctional proteins, functioning as a source of amino acids for the plant during germination and subsequent growth and at the same time being toxic to fungi and insects (Macedo *et al.*, 1993; Sales *et al.*, 2000; Shutov *et al.*, 1995).

Thaumatin-like proteins

Osmotin and thaumatin-like (TL) proteins are basic, 24-kDa proteins belonging to the PR-5 family and sharing significant sequence homology to thaumatin, a sweet-tasting (to humans) protein from the South African Ketemfe berry bush (*Thaumatococcus*

danielli) (van der Wel and Loeve, 1972). TL proteins have been detected in a vast number of plants. For example, a 24-kDa TL protein is abundantly expressed in grapevine fruits not only in a berry- and ripening-specific manner (Tattersall *et al.*, 1997) but also in response to *Erysiphe necator* infection (Monteiro *et al.*, 2003a). TL proteins have been isolated from the intercellular fluid of lupin (*Lupinus albus*) leaf, stem and root tissues (Regalado and Ricardo, 1996) and from the intercellular fluid of chickpea leaves (Hanselle *et al.*, 2001). TL proteins are also produced in plants under different stress conditions (Zhu *et al.*, 1995).

Osmotin and TL proteins induce fungal cell leakiness presumably through a specific interaction with the plasma membrane that results in the formation of transmembrane pores (Kitajima and Sato, 1999; Roberts and Selitrennikoff, 1990). These proteins have also been reported to possess β-1,3-glucanase activity (Grenier *et al.*, 1999) or bind to actin (Takemoto *et al.*, 1997). Tobacco osmotin stimulates a mitogen-activated protein kinase, subverting a signal transduction pathway to enhance fungal cell susceptibility (Grenier *et al.*, 1999; Yun *et al.*, 1998). The proteins exhibit antifungal activity *in vitro* (Liu *et al.*, 1994; Melchers *et al.*, 1993; Woloshuk *et al.*, 1991) and show enhanced lytic activity when tested in combination with chitinases and/or β-1,3-glucanases (Lorito *et al.*, 1996). Furthermore, the simultaneous presence of both osmotin and TL protein from grapevine displays a synergistic antifungal effect (Monteiro *et al.*, 2003a).

Defensins/thionins

Defensins and thionins are families of low-molecular-mass (about 5 kDa), cysteine-rich peptides (45–54 amino acid residues in length) that form a prominent group of membrane-acting proteins found in mammals, insects, plants and fungi (Theis and Stahl, 2004). Plant defensins (PR-12 proteins) and thionins (PR-13 proteins), present in both monocotyledonous and dicotyledonous plants, are toxic to fungi (Bohlmann, 1994; Broekaert *et al.*, 1995; Evans and Greenland, 1998). Broekaert *et al.* (1997) suggested that these peptides play a role in protecting seeds from infection by pathogens.

The mode of action of plant defensins has not yet been properly elucidated. These peptides induce a prompt potassium efflux, calcium uptake, alkalinization of the medium and membrane potential changes in *Neurospora crassa* (Theis and Stahl, 2004). In contrast to other membrane-acting proteins, plant defensins do not form pores on artificial membranes (Thevissen *et al.*, 1996). Indeed, selective calcium uptake through activated ion channels, but not membrane permeabilization, is thought to be a major component of plant defensin antifungal action (Theis and Stahl, 2004). A specific, high-affinity binding site for a plant defensin on *N. crassa* has been found (Thevissen *et al.*, 1997). The defensin Dm-AMP1 from dalhia (*Dalhia merckii*) has been shown to interact specifically with a sphingolipid from *Saccharomyces cerevisiae*. Yeast mutants lacking the sphingolipid are highly resistant to the defensin (Thevissen *et al.*, 2000).

Differential antifungal activity has been detected among structurally related plant defensins. MsDef1, a seed defensin from alfalfa (*Medicago sativa*), inhibits the growth of *Fusarium graminearium in vitro*. However, MtDef2 from *Medicago trunculata*, which shares 65% amino acid sequence identity with MsDef1, lacks antifungal activity towards *F. graminiarum* (Spelbrink *et al.*, 2004).

Cyclophilin-like proteins

Cyclophilins are a highly conserved group of proteins that function as intracellular receptors for cyclosporin. Mungin, for example, is an 18-kDa protein present in mung bean which shows a significant homology to cyclophilins and inhibits α- and β-glycosidases *in vitro* (Ye and Ng, 2000). Unguilin, another example, is an 18-kDa cyclophilin-like protein isolated from the seeds of the black-eyed pea (*Vigna unguiculata*) that exhibits antimitogenic, antiviral and antifungal activities towards fungi, including *Coprinus comatus*, *Mycosphaerella arachidicola* and *Botrytis cinerea.*

Glycine/histidine-rich proteins

These are antifungal insect proteins whose mechanism of action remains to be elucidated. Glycine and histidine may comprise up to 80% of their amino acid residues.

Ribosome-inactivating proteins

A prominent intracellular target for antifungal proteins is ribosomes. RIPs are a group of cytotoxic *N*-glycosidases that specifically cleave nucleotide N–C glycosidic bonds (Park *et al.*, 2004b). They are enzymes with RNA *N*-glycosidase activity, which depending on their specificity, can inactivate non-specific or foreign ribosomes, thereby shutting down protein synthesis (Punja, 2001). Regardless of the activity type, ribosome damage (i.e. depurination) occurs at the sarcin/ricin loop, a highly conserved sequence of the 28S rRNA gene (Endo and Wool, 1982). Some RIPs consist of a single polypeptide chain (type I), whereas others are dimeric proteins with one catalytic polypeptide chain and another responsible for translocation into cells via recognition of protein receptors (type II). In type III RIPs, both domains are contained in a single polypeptide (Peumans *et al.*, 2001; Stirpe *et al.*, 1992).

Plant RIPs inhibit mammalian, plant, fungal and bacterial protein syntheses, either *in vivo* or *in vitro* (Iglesias *et al.*, 1993). How plants protect themselves from their own RIPs has been under investigation. For example, type I RIP from the endosperm of cereal grains does not act on plant ribosomes but affects foreign ribosomes such as those of fungi (Hartley *et al.*, 1996; Stirpe *et al.*, 1992).

Although RIPs were first identified more than 100 years ago, their biological function(s) still remains open to speculation (Park

et al., 2004b). Several independent studies suggest that their antimicrobial activity and their inhibitory mechanism against HIV replication are separate from their host-ribosome-inactivating activity. It has also been reported that their ribosome-inactivating activity does not account for their cytotoxicity (Park *et al.*, 2002b). Increasing evidence suggests that RIPs can target non-ribosomal substrates. Thus, for example, RIPs may regulate protein expression by targeting mRNA instead of ribosomes (Park *et al.*, 2004b).

Examples of plant RIPs are ricin from castor bean (*Ricinus communis*) (Endo *et al.*, 1987), ME1+2 from *Mirabilis expansa* (Vivanco *et al.*, 1999), RIP1 from maize (Nielsen *et al.*, 2001), PAP-H from pokeweed (*Phytolacca americana*) (Park *et al.*, 2002a), α- and β-pisavins from pea (*Pisum sativum*) seeds (Lam *et al.*, 1998) and ebulin1, a type II RIP from *Sambucus ebulus* (Girbes *et al.*, 1993). Hispin is a 21-kDa RIP from hairy melon seeds (Ng and Parkash, 2002), whereas luffacylin is an arginine- and glutamate-rich RIP from loofah (*Luffa cylindrical*) (Parkash *et al.*, 2002). A 30-kDa RIP was isolated from dehursked barley grains (Roberts and Selitrennikoff, 1986).

Lipid transfer proteins

Plant lipid transfer proteins (LTPs; PR-14) are small, basic proteins, stabilized by four disulphide bonds, which transfer phospholipids between membranes. LTPs contain typically an internal, tunnel-like hydrophobic cavity that runs through the molecule (Cheng *et al.*, 2004; Selitrennikoff, 2001). The mechanism responsible for their antifungal activity remains unknown, although it was suggested that these proteins insert themselves into the fungal cell membrane with their central hydrophobic cavity forming a pore, allowing efflux of intracellular ions and leading to fungal cell death (Selitrennikoff, 2001). Unlike the non-specific LTPs (nsLTPs) from raddish (*Raphanus sativus*) and maize seeds, Ace-AMP1, a 10-kDa LTP from onion (*Allium cepa*) seeds, is incapable of phospholipid transfer from liposomes to mitochondria (Ng, 2004). Expression of Ace-AMP1 in transgenic wheat has been shown to enhance antifungal activity and defence responses (Roy-Barman *et al.*, 2006). A putative LTP from Arabidopsis has been implicated in the transport of the systemic signal in SAR, leading to enhanced resistance to subsequent attack by a broad range of normally virulent pathogens (Maldonado *et al.*, 2002). Other LTPs have recently been characterized in the seeds of cowpea and motherwort (*Leonurus japonicus*) (Carvalho *et al.*, 2006; Yang *et al.*, 2006).

Killer proteins

Killer proteins or killer toxins are secreted by several yeasts and bind to specific surface receptors in sensitive fungal cells. They are subsequently internalized and can disrupt cell wall synthesis, DNA synthesis and K^+ channel activity, inhibit β-1,3-glucan synthesis and arrest the cell cycle, leading to inhibition of fungal growth and fungal cell death (Ahmed *et al.*, 1999; Eisfeld *et al.*, 2000; Kimura *et al.*, 1997, 1999; Suzuki and Shimma, 1999). A *Kluyveromyces* yeast killer toxin has been shown to share homology with chitinases from plant, yeast and bacterial sources (Bradshaw, 1990; Kuranda and Robbins, 1991; Stark *et al.*, 1984). Perhaps, the secreted *Saccharomyces* chitinase exhibiting homology with the killer toxin plays a dual role by selectively modifying the yeast cell wall and also suppressing the growth of other micro-organisms (Kuranda and Robbins, 1991).

Protease inhibitors

One of the major classes of proteins present in some plant tissues, with correspondence to the PR-6 family, include inhibitors of metal, aspartic, serine and cysteine proteases (Kassel, 1970). Inhibitors of serine proteases, such as trypsin and chymotrypsin, are sometimes considered bifunctional proteins because they also inhibit other enzymes such as α-amylase and fungal growth (Selitrennikoff, 2001). A 7.5-kDa antifungal, Bowman–Birk-type trypsin–chymotrypsin inhibitor was isolated from broad bean (*Vicia faba*) seeds (Ye *et al.*, 2001a), whereas an antifungal, sporamin-type trypsin inhibitor was purified from wampee (*Clausena lansium*) seeds (Ng *et al.*, 2003). Plant antifungal cysteine protease inhibitors, termed phytocystatins, have been isolated from many plants (Joshi *et al.*, 1998; Park *et al.*, 2000; Soares-Costa *et al.*, 2002).

Other antifungal proteins

As new antifungal proteins continue to be discovered, an increasing number of them do not fall clearly into any of the previous 12 classes. Among them are well-known proteins such as lectins, ribonucleases, deoxyribonucleases and peroxidases. Selected examples are viridin, present in the culture medium of *Trichoderma viride*, and snakin-1 isolated from potato (*Solanum tuberosum*).

Lectins with antifungal activity have been reported in the red kidney bean (*Phaseolus vulgaris*) (Ye *et al.*, 2001b), stinging nettle (*Urtica dioica*) (Broekaert *et al.*, 1989), potato tuber (Gozia *et al.*, 1995) and slender amaranth (*Amaranthus viridis*) (Kaur *et al.*, 2006). A role in plant defence against fungi was proposed for the cotyledonary *Lutzelburgia auriculata* agglutinin (LAA). This lectin is located in the periphery of the cotyledon and is released during germination into the surrounding medium. Inclusion of LAA in the culture medium inhibits growth of *Colletrotichum lindemuthianum*, *Fusarium solani*, *Aspergillus niger* and *Saccharomyces cerevisiae*. LAA was found to bind reversibly to yeast cells (Melo *et al.*, 2005).

Quinqueginsin and panaxagin are two antifungal proteins exhibiting ribonuclease activity that have been purified from the roots of the American ginseng (*Panax quinquefolium*) and the

Chinese ginseng (*Panax ginseng*), respectively. Quinqueginsin possesses specific ribonucleolytic activity towards poly C (Ng and Wang, 2001; Wang and Ng, 2000b). In addition, a deoxyribonuclease with antifungal activity was extracted from asparagus (*Asparagus officinalis*) seeds (Wang and Ng, 2001), whereas an antifungal peroxidase was reported in french bean (*Phaseolus vulgaris*) legumes (Ye and Ng, 2002b).

The storage 2S albumin proteins have also been reported to display antifungal activity in passion fruit (*Passiflora edulis*), radish (*Raphanus sativus*) and oilseed rape (*Brassica napus*) (Agizzio *et al.*, 2003; Pelegrini *et al.*, 2006; Terras *et al.*, 1993). In addition, the puroindolines, endosperm-specific proteins involved in wheat seed hardness or texture (Morris, 2002), have been shown to play a role in plant defence (Giroux *et al.*, 2003). Indeed, rice plants, which normally lack puroindolines, were transformed to express constitutively puroindoline genes. The transgenic rice showed significantly increased tolerance to *Magnaporthe grisea*, with a 29–54% reduction on symptoms, and to *Rhizoctonia solani*, with an 11–22% reduction in symptoms (Krishnamurthy *et al.*, 2001).

Ginkbilobin is a 13-kDa antifungal protein from maidenhair tree (*Ginkgo biloba*) seeds with sequence similarity to embryo-abundant protein (Wang and Ng, 2000a). Hypogin is an allergen-like, antifungal protein from peanut (*Arachis hypogaea*) with sequence similarity to peanut allergen (Ye and Ng, 2000). Pisumin, an antifungal protein with a novel *N*-terminal sequence, and sativin, a miraculin-like antifungal protein, have been characterized in legumes of the sugar snap pea (*Pisum sativum* var. macrocarpon) (Ye *et al.*, 2000b).

Germin-like oxalate oxidases (proposed PR-15 and PR-16 families) are stable glycoproteins that were initially discovered in cereals. They are present during seed germination and are induced in response to fungal infection (Dumas *et al.*, 1995; Zhang *et al.*, 1995). The hydrogen peroxide produced as a result of their activity upon the substrate oxalic acid induces plant defence responses and enhances cell-wall strengthening (Brisson *et al.*, 1994; Mehdy, 1994).

Antifungal peptides

Although there is not a generalized consensus concerning where a peptide ends and a protein begins, it may be arbitrarily assumed that proteins possess molecular masses greater than 5 kDa or about 50 amino acid residues in length. Therefore, some groups of antifungal compounds, such as plant defensins, include both proteins and peptides.

An enormous variety of peptides with antifungal activity are produced by mammals (e.g. defensis, protegrins, gallinacins, tritrpticin, lactoferricin, and BPI protein domain III analogues), insects (e.g. cecropins, drosomycin, antifungal peptide, holotricin 3 and thanatin), amphibians (e.g. magainins and dermaseptin), bacteria and fungi (e.g. iturins, syringomycins and related peptides, nikkomycins, polyoxins, echinocandins and echinocandin analogues, pneumocandins and pneumocandin analogues, aculeacins, mulundocandins, aureobasidins, *Bacillus licheniformis* peptides, schizotrin A, cepacidines, leucinostatin-trichopolyn group, and helioferins) and plants (e.g. plant defensins, lipid transfer proteins, zeamatin and cyclopeptides) (De Lucca and Walsh, 1999).

Antifungal peptides are classified according to their mode of action. One large group, including many amphipathic lytic peptides, act by cell lysis, which operate via a number of different mechanisms (Shai, 1995). Another group interferes with cell wall synthesis or with the biosynthesis of essential cell components, such as glucan or chitin (Debono and Gordee, 1994).

Antifungal peptides are continuously being discovered in plants. Thus, for example, cicerin and arietin were detected in the seeds of chickpea (Ye *et al.*, 2002). The 8-kDa angularin was purified from red beans (Ye and Ng, 2002c). A 30-amino acid residue peptide containing six cysteine and seven glycine residues and exhibiting sequence homology to the chitin-binding domain of chitin-binding chitinases and a higher affinity to chitin than chitin-binding chitinases has been isolated from the intercellular washing fluid from sugar beet (*Beta vulgaris*) leaves (Kristensen *et al.*, 2001; Nielsen *et al.*, 1997). A 1.244-kDa chitin-binding peptide with an amino acid sequence and a cystein/glycine-rich chitin-binding domain typical of many chitin-binding proteins was characterized in *Ginkgo biloba* leaves (Huang *et al.*, 2000).

Plant defensins that are not related to either mammalian or insect defensins have been characterized in plant tissues. This is the case, for example, of Ib-AMP$_3$, a highly basic, icosapeptide produced by garden balsam (*Impatiens balsamina*) (Tailor *et al.*, 1997). Zeamatin is a 27-amino-acid residue peptide produced by maize seeds. However, peptides from the zeamatin family are also present in oat, sorghum and wheat seeds (De Lucca and Walsh, 1999). Examples of plant cyclopeptides, recently reviewed by Tan and Zhou (2006), are frangufoline, amphibine H, rugosanines A and B, and nummularines B, K, R and S (Panday and Devi, 1990).

STRATEGIES EMPLOYED BY FUNGI TO AVOID PLANT DETECTION OR DEFENCE

Emerging evidence demonstrates that the molecular interaction between plant and pathogen is far more elaborate than the straightforward production of attack molecules by the pathogen and the corresponding response of defensive molecules by the host. Plant pathogens use several strategies to avoid detection by the host plant or to escape the plant defence responses.

One of the strategies involves protection of fungal structures from plant defence mechanisms. Thus, for example, the attack of fungal cell walls by plant chitinases is an important plant defence to fungal infection because it liberates elicitor-active chitin oligomers and weakens the fungal cell wall (van den Burg *et al.*, 2003). The antifungal activity of most plant chitinases derives

from the presence of a non-catalytic, plant-specific chitin-binding domain (ChBD). The race-specific elicitor avr4, an extracellular avr protein produced by the leaf mould fungus *Cladosporium fulvum* and detected by the tomato Cf-4 LRR-RLP protein, protects the fungus against degradation by tomato chitinases because it contains a novel type of ChBD (Jones and Takemoto, 2004). However, the binding site of avr4 is larger than that of a plant ChBD. In other words, avr4 interacts only with chitotriose whereas a plant ChBD also interacts with the monomer *N*-acetyl-glucosamine. Binding additional avr4 molecules to chitin occurs through positive cooperative protein–protein interactions. These observations suggest that avr4 shields fungal cell wall chitin from the action of plant chitinases (van den Burg *et al*., 2004).

During invasive growth of biotrophic rust fungi, chitin is exclusively present in the cell walls of exterior infection structures, i.e. germ tubes and appressoria. Instead of this elicitor-active molecule, the surface hyphae that grow within host leaves contain chitosan, a deacetylation product of chitin possibly generated by the enzymatic activity of a differentiation-induced fungal-chitin deacetylase (Deising and Siegrist, 1995; El Gueddari *et al*., 2002). The observation that chitosan lacks elicitor activity led Schulze-Lefert and Panstruga (2003) to speculate that the 'wolf intrudes in sheep's clothing'.

The tobacco pathogen *Alternaria alternata* synthesizes and secretes the reactive oxygen quencher mannitol as a means of suppressing reactive oxygen-mediated plant defences (Jennings *et al*., 2002). Interestingly, the non-mannitol-containing host tobacco plants respond by expressing a pathogen-induced mannitol dehydrogenase that catabolizes mannitol of fungal origin. Indeed, constitutive expression of a celery (*Apium graveolens*) mannitol dehydrogenase cDNA in tobacco plants conferred enhanced resistance to *A. alternata*, but not to the non-mannitol-producing fungal pathogen *Cercospora nicotianae* (Jennings *et al*., 2002).

Another strategy is associated with inhibition of elicitor-induced plant defence responses. Oligomers of galacturonic acid released from plant cell walls during pathogenesis may function as plant-derived suppressors of defence responses. In fact, the simultaneous addition of oligogalacturonides and the glycoproteogalactan elicitor isolated from germ tubes of the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* suppresses the elicitor-induced disease resistance reactions in wheat leaves (Moerschbacher *et al*., 1999).

Yet another strategy concerns the suppression of plant defences. An increasing body of evidence suggests that infecting fungal pathogens may modulate host gene expression to their own benefit, either by suppressing inducible plant defence responses in physical proximity to infection sites and/or by inducing specific host genes required for infection. An example of fungal-induced plant defence suppression is provided by the infection of barley coleoptile cells by the grass powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. Barley cells penetrated by the fungus or in close proximity to haustoria exhibit induced susceptibility to subsequent attack by the non-host pathogen *Erysiphe pisi* or by a second challenge with *B. graminis* (Kunoh *et al*., 1985, 1991). These observations suggest that *B. graminis* suppresses defence gene activation of barley cells by an as yet unknown mechanism that is effective against race-specific, race-non-specific and non-host resistance (Schulze-Lefert and Panstruga, 2003).

CONCLUDING REMARKS

The continuous increase in the human population and in public concern over the generalized use of chemical fungicides, associated with the increasing number of obsolete fungicides that derive from the development of fungal resistance, demand alternative ways for disease control. These may include the development of new, effective and environmentally friendly fungicides. However, emerging evidence suggests that during a plant–fungus interaction, the pathogen may take over selected aspects of plant gene expression to its own benefit. Therefore, it is expected that the fungus may induce the expression of some components required for the infection or development processes or repress components of the host defence system. In this context, a detailed understanding of the molecular events that take place during a plant–pathogen interaction is a prerequisite for boosting the natural inherent defences of plants, and to transfer defensive traits into the genome of economically important crops.

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