Abstract: Plants produce potent constitutive and induced antifungal compounds to complement the structural barriers to microbial infection. Approximately 250,000 – 500,000 plant species exist, but only a few of these have been investigated for antimicrobial activity. Nevertheless, a wide spectrum of compound classes have been purified and found to have antifungal properties. The commercial potential of effective plant-produced antifungal compounds remains largely unexplored. This review article presents examples of these compounds and discusses their properties.

Key words: antifungal, peptides, phytopathogenic, plants, proteins.

Introduction
Floristic analyses indicate that approximately 250,000 – 500,000 plant species currently exist (Tivy 1993; Borris 1996). Though hundreds of plant-produced antifungals have been identified, few are commercially used. However, plants should be an excellent source of potent antifungals, since they are exposed to a wide array of phytopathogenic fungi present in their environment and have had to develop antifungal compounds to survive.

Phytopathogenic fungi must overcome complex defenses (Bell 1981; Borgmeyer et al. 1992; Linthorst 1991). These defenses include a cell wall, composed of compounds such as lignin, tannins, phenols, and cellulose, which presents a physical barrier to inhibit microbial infections (Bell 1981; Bol and Linthorst 1990). Though plants lack an immune system, they do produce constitutive and (or) induced proteins in response to fungal infection (Broekaert et al. 1997; Garcia-Olmedo et al. 1998; Selitrennikoff 2001). Research continues to discover novel antifungal plant-produced peptides and proteins with potential utility in agriculture, medicine, food processing, and other areas where fungal inhibiting and (or) fungicidal compounds are needed.

Proteinaceous plant antifungals
Plants respond to infection and stress by synthesizing a variety of host plant proteins. Peptides are generally defined as proteins of no more than 100 amino acid residues in length whose molecular weight is not greater than 10,000. In contrast, the term “plant protein” is defined as proteins greater than 100 amino acid residues with molecular weights greater than 10,000. A large number of both protein types have been purified and studied. Below is a brief review of these proteins.

Plant peptides
Plants produce a number of cysteine-rich, antifungal peptide classes that are based on amino acid sequence homology and are commonly found in seeds (see Table 1). They are both inducible and constitutive and they differ in size and structure. Plant antifungal peptides include the \( \alpha \)-defensins, lipid transfer proteins (LTPs), thionins, hevein- and knottin-type peptides, the cyclopeptide alkaloids, and other unique peptide groups (Broekaert et al. 1997; Garcia-Olmedo et al. 1998). Several of these peptide families (defensins, LTPs, and thionins) are classified as pathogenesis-related (PR) proteins, which are defined as proteins genetically coded for by
<table>
<thead>
<tr>
<th>Peptide type (peptide family)</th>
<th>Source</th>
<th>Active against</th>
<th>Mode of action</th>
<th>In vitro MIC (µg/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-AMP1 (hevein)</td>
<td>Amaranthus caudatus</td>
<td>Fusarium culmorum</td>
<td>Binds chitin</td>
<td>2.0</td>
<td>Broekaert et al. 1992</td>
</tr>
<tr>
<td>Ace-AMP1 (ns-LTP)</td>
<td>Allium cepa</td>
<td>Fusarium oxysporum</td>
<td>Phospholipid binding*</td>
<td>10.0</td>
<td>Cammue et al. 1995; Tassin et al. 1998</td>
</tr>
<tr>
<td>Ah-AMP1 (α-defensin)</td>
<td>Aesculus hippocastanum</td>
<td>Fusarium culmorum</td>
<td>Receptor mediated*</td>
<td>0.7 (IC50)</td>
<td>Terras et al. 1993</td>
</tr>
<tr>
<td>Amphibine H (cyclopeptide)</td>
<td>Zizyphus xylopyra</td>
<td></td>
<td>Unknown</td>
<td>5.0</td>
<td>Panday and Desi 1990</td>
</tr>
<tr>
<td>Beta vulgaris</td>
<td></td>
<td>Cercospora beticola</td>
<td>Inhibits hyphal tip growth</td>
<td>4.0</td>
<td>Kragh et al. 1995</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td></td>
<td>Fusarium solani</td>
<td>Pore formation*</td>
<td>174.0</td>
<td>Molina et al. 1993</td>
</tr>
<tr>
<td>Hs-AFP1 (α-defensin)</td>
<td>Heuchera sanguinea</td>
<td>Fusarium moniliforme</td>
<td>Receptor mediated*</td>
<td>125.0</td>
<td>Thevissen et al. 1997</td>
</tr>
<tr>
<td>Ib-AMP1 (Ib-AMP)</td>
<td>Impatiens balsamina</td>
<td>Fusarium culmorum</td>
<td>Unknown</td>
<td>2.0 (IC50)</td>
<td>Nielsen et al. 1997</td>
</tr>
<tr>
<td>IWF4 (hevein)</td>
<td>Beta vulgaris</td>
<td>Cercospora beticola</td>
<td>Unknown</td>
<td>2.0 (IC50)</td>
<td>Nielsen et al. 1997</td>
</tr>
<tr>
<td>MBP-1</td>
<td>Phytolocca americana</td>
<td>Fusarium oxysporum</td>
<td>Spore germination</td>
<td>40.0</td>
<td>Molina et al. 1995</td>
</tr>
<tr>
<td>Pn-AMP-1 (hevein-like)</td>
<td>Actin depolarisation</td>
<td>Fusarium oxysporum</td>
<td>Unknown</td>
<td>50.0</td>
<td>Koo et al. 1998</td>
</tr>
<tr>
<td>Rs-AFP1 (α-defensin)</td>
<td>Raphanus sativus</td>
<td>Fusarium moniliforme</td>
<td>Receptor mediated*</td>
<td>125.0</td>
<td>De Lucca et al. 1999</td>
</tr>
<tr>
<td>Snakin-1</td>
<td>Solanum tuberosum</td>
<td>Fusarium solani</td>
<td>Pore formation</td>
<td>138.0</td>
<td>Segura et al. 1999</td>
</tr>
<tr>
<td>Wβ (thionin)</td>
<td>Triticum aestivum</td>
<td>Fusarium graminearum</td>
<td>Spore germination</td>
<td>60.0</td>
<td>Duvick et al. 1992</td>
</tr>
</tbody>
</table>

The table above provides examples of plant antifungal peptide families, along with their sources, the fungi they are active against, the mode of action, and their minimum inhibitory concentrations (MIC) in µg/mL. Note: MIC, minimum inhibitory concentration; IC50, concentration producing 50% growth inhibition.

α-Defensins

α-Defensins, present in seeds or leaves, have complex structures containing disulfide-linked cysteines in a triple-stranded antiparallel β-sheet with only one α-helix (Osborn et al. 1995; Terras et al. 1992, 1993a, 1995) and are homologous to those produced by human and rabbit neutrophils (Fant et al. 1997, 1998). Plant α-defensins are considered PR proteins and are classified as members of the PR-12 group (Van Loon and van Strien 1999). These peptides are present (Terras et al. 1992, 1993a, 1995; Osborn et al. 1995) in the seeds or leaves of Heuchera sanguinea (Hs-AFP1), Raphanus sativus (Rs-AFP1, through Rs-AFP2), Aesculus hippocastanum (Ah-AMP1), Citrolea ternaeta (CT-AMP1), and Dahlia merckii (DM-AMP1). Plant α-defensins do not form ion-permeable pores in artificial membranes but may act via a receptor-mediated mechanism (Thevissen et al. 1996), as suggested by the observation that the binding site for Hs-AFP1 resides on fungal plasma membranes (Thevissen et al. 1997). Seed-borne α-defensins are released upon germination after seed coat disruption and create a microenvironment in which fungal growth was suppressed (Terras et al. 1995). These peptides apparently act on the early stage of hyphal development. The plant defensins Hs-AFP1 and Rs-AFP2 are lethal to the germinating conidia but not the nongerminated conidia of Aspergillus flavus and Fusarium moniliforme, indicating that the early-stage developing hyphae but not dormant conidia have binding sites for these defensins (De Lucca et al. 1999). The activity of plant defensins is reduced by an increase in ionic strength, especially by divalent cations (Broekaert et al. 1997; Osborn et al. 1995; Terras et al. 1992, 1993a).

Thionins

The thionin peptide family are antimicrobial plant proteins containing 45–47 amino acids with two antiparallel α-helices and an antiparallel double-stranded β-sheet with six or eight cysteines forming disulphide bridges (Fant et al. 1997; Briux et al. 1993a, 1993b, 1995). Thionins are classified as PR-13 proteins (Van Loon and van Strien 1999). Thionins occur in seeds, stems, roots, and leaves of several plant species and are grouped according to the number of cysteines present. Antifungal activity results from direct protein–membrane interactions (Bohlmann and Apel 1987; Thevissen et al. 1996) by electrostatic interaction of the positively charged thionin with the negatively charged phospholipids in fungal membranes, and this results in pore formation or a specific interaction with a certain lipid domain (Florack and Stiekema 1994). Type 1 thionins (Wa1, Wa2, and Wβ) isolated from wheat endosperms and barley (Bx and Bβ) and Type 2 thionins (BLa, BLb, and BLc) from barley leaves inhibit the growth of a number of phytopathogenic fungi, including Fusarium solani, with EC50 values between 1×10⁻⁶ and 4×10⁻⁶ mol/L range (Molina et al. 1993). The addition of other cysteine-rich peptides, such as seed storage 2S albums, protease inhibitors, LTPs, and wheat puroindolines, enhance the antifungal properties of each of
the thionins (Blochet et al. 1993; Molina et al. 1993; Terras et al. 1993b). In contrast, the antifungal activity of this peptide family is inhibited by Ca^{2+} at concentrations above 5 mmol/L but not by Mg^{2+} or Br^{2-} at levels up to 10 mmol/L or by monovalent cations at concentrations up to 50 mmol/L (Terras et al. 1992).

The cysteine-rich antifungal peptides AX1 and AX2 are present in sugar beet leaves. They are monomeric, basic proteins consisting of 46 amino acid residues, of which eight are cysteines, and are related to the thionins (Kragh et al. 1995). Immunoblotting and immunohistology show that the AX peptides are present in high concentrations extra-cellularly in the cell walls and in necrotic lesions of sugar beet leaves infected with Cercospora beticola, suggesting a role in antifungal defense (Kragh et al. 1995). At concentrations ≤ 1 μmol/L, AX1 and AX2 prevent the growth of the plant pathogens C. beticola and Botrytis cinerea but have little or no activity against bacterial growth. AX2 reduces the longitudinal hyphal growth of C. beticola though the fungus develops side branch growth (Kristensen et al. 1999). Recombinant AX2, despite being correctly expressed and folded, was much less active than the native form (Kristensen et al. 1999). The recombinant AX2 contained an additional N-terminal arginine that introduced an additional positive charge to the molecule. This additional positive charge may weaken the ionic interaction between the peptide and its putative receptor. Another possibility is that the extra charge affects the three-dimensional structure of AX2, which in combination with the change in charge, could affect the fungal inhibition properties of AX2 (Kristensen et al. 1999).

Plant nonspecific LTPs

Plant nonspecific LTPs (ns-LTPs) are basic proteins, 9–10 kDa in size, that are known for their ability to enhance in vitro the intermembrane exchange and (or) transfer of various polar lipids, such as phospholipids and glycolipids (Arondel and Kader 1990; Douliez et al. 2001). Their antifungal mode of action is not yet known, though they may insert themselves in fungal membranes and form a pore resulting in an efflux of intracellular ions culminating in cell death (Selitrennikoff 2001; Van Loon and van Strien 1999). A common structure for several ns-LTP molecules (except Ace-AMP1) believed to play a role in their antifungal properties is the presence of a continuous cavity, which is a binding site for lipids, that passes through each molecule (Gincel et al. 1994; Shin et al. 1995). These compounds, which are classified as PR-14 proteins (Van Loon and van Strien 1999), are much larger than and differ in structure from thionins and plant defensins. Molina et al. (1993) demonstrated that homologous ns-LTPs (Cw18, Cw20, Cw21, and Cw22) present in barley leaves inhibit the growth of F. solani.

Several related ns-LTP antifungal compounds, IWF1, IWF2, and IWF5, are present in the intercellular washing fluid of sugar beet leaves (Nielsen et al. 1996; Kristensen et al. 2000b). The first two are nearly identical to each other and are basic, monomeric proteins of 91 and 89 amino acid residues (Nielsen et al. 1996). IWF5 is a monomer in both its reduced and unreduced states, having 92 amino acids (eight are cysteines), and is 47% identical to IWF1 (Kristensen et al. 2000b). IWF5 has a tunnel-like hydrophobic cavity that could be a potential binding site for lipids (Gincel et al. 1994; Shin et al. 1995). IWF5 is similar to the antifungal peptides AX1 and AX2 (also present in sugar beet leaf washings and discussed earlier) in that at low concentrations it inhibits the growth of C. beticola, as do IWF1 and IWF2 (Nielsen et al. 1996; Kristensen et al. 2000b).

Ace-AMP1 is a nonhaemolytic peptide present in onion seeds, with 93 amino acid residues and four disulfide bridges (Tassin et al. 1998). This peptide shares 76% of the residues conserved among all ns-LTPs and is unusually rich in arginine (20% of the amino acids), but as opposed to the ns-LTPs, Ace-AMP1 is unable to transfer phospholipid from liposomes to mitochondria. This molecule differs from IWF5 in that the continuous hydrophobic cavity, which runs through the center of IWF5 and is believed to bind lipids, is absent (Tassin et al. 1998). It also differs from members of the ns-LTP group in that it does not bind phospholipids in solution but does interact with phospholipid membranes of artificial liposomes (Tassin et al. 1998). Ace-AMP1 inhibits the growth of 12 phytopathogenic fungi, including Fusarium oxysporum, at concentrations ≤10 μg/mL. Cations near physiological ionic strength do not, or only weakly, affect its antifungal properties.

Hevein and hevein-like peptides

Hevein, a small 4.7-kDa, cysteine-rich, chitin-binding peptide, is present in the luteoid bodies of rubber tree (Hevea brasiliensis) latex (Archer 1960; Walujono et al. 1975). This peptide is resistant to heat (90 °C for 10 min) and is found in the bottom fraction of the latex and strongly resembles the chitin-binding lectin from the stinging nettle, Urtica dioica (Van Parijs et al. 1991). Hevein, wheat germ agglutinin, and the stinging nettle lectin share amino acid sequence homology, so it is not surprising that these three peptides act in the same way, that is, by binding chitin (Van Parijs et al. 1991). Hevein inhibits the hyphal growth of fungi by binding to chitin.

Hevein-like peptides are small (43 residues) chitin-binding peptides. While hevein is a rather weak antifungal (Van Parijs et al. 1991), the hevein-like peptides inhibit the growth of Alternaria brassicicola, Ascochyta pisi, and Fusarium culmorum at doses lower than those for other known chitin-binding proteins. Ac-AMP1 and Ac-AMP2 are members of the hevein-like peptide family, with 29 and 30 amino acid residues, respectively (Broekaert et al. 1992), they share sequence homology to the cysteine- and (or) glycine-rich domain of chitin-binding proteins (see below), and they both reversibly bind to chitin. Ac-AMP1 and Ac-AMP2 are strongly antagonized by cations.

Seeds of Pharbitis nil contain two α-hevein analogs, Pn-AMP1 and Pn-AMP2 (with 41 and 40 amino acid residues, respectively), that cause leakage of cytoplasmic materials by attaching to hyphal tips and septum (Koo et al. 1998). Both are highly basic molecules exhibiting potent antifungal activity against both chitin-containing and non-chitin-containing fungi and are considered the first hevein-like peptides to show fungicidal effects similar to those of thionins. The Pn-AMPs differ in mode of action from the other members of the hevein group. The latter bind to chitin, whereas the Pn-AMPs cause rapid depolarization of the actin cytoskeleton within 15 min (Koo et al. 2004). Pn-AMP1 and Pn-AMP2
have been constitutively expressed in tomato and have enhanced resistance against both nonchitinoid and chitin-containing fungi (Lee et al. 2003). The Pn-AMPs are present during seed maturation and germination but not in the aerial vegetative plant parts (Koo et al. 1995).

IWF4 is one of a group of antifungal peptides present in the interstitial wash fluid of sugar beet leaves. It is considered a hevein-like peptide because of its homology to the hevein-type Ac-AMP peptides that bind to chitin (Nielsen et al. 1997; Koo et al. 1998). IWF4 has six cysteine and seven glycine residues and inhibits the growth of *C. beticola* with an IC$_{50}$ of $\leq$ 2 µg/mL, which is comparable to the Ac-AMP peptides (Koo et al. 1998). The mature IWF4 and Ac-AMP protein families resemble each other in size, pI, amino acid sequence, chitin-binding ability, and antifungal potentials (Nielsen et al. 1997). However, IWF4 differs from Ac-AMP$_2$ in that it is present in vegetative, aerial tissues and is not induced by pathogens, whereas Ac-AMP$_2$ is present in mature seeds of amaranth only, not vegetative tissues, and is induced by pathogen infection or other stress factors (Nielsen et al. 1997).

Two novel antifungal peptides, EAFP1 and EAFP2, contain 41 amino acid residues, are present in the bark of the tree *Eucommia ulmoides*, and share core amino acid sequences with hevein (Huang et al. 2002). This tree produces a latex similar to that of the rubber tree, *Hevea brasiliensis* (Huang et al. 2002), which produces hevein. Both peptides show characteristics of a hevein domain and exhibit chitin-binding properties similar to other hevein-like peptides (Huang et al. 2002). They are also similar to hevein in that they are heat resistant. EAFP1 and EAFP2 are the first plant-produced, antifungal hevein-like peptides isolated that contain 10 cysteines that are paired and form five disulfide bridges (Huang et al. 2002). In comparison, hevein and the other hevein-like peptides have only three or four disulfide bridges that stabilize a chitin-binding domain (Huang et al. 2004). The unique disulfide bond Cys7–Cys37 confers on EAFP2 a more defined amphiphatic character than that of hevein, which also does not have the cationic face and amphipathic topology of EAFP2 (Huang et al. 2004). EAFP1 and EAFP2 are similar to the Pn-AMP peptides in that they are active against both chitinoid and nonchitinoid fungi, whereas hevein is only active against chitinoid fungi. Structural analysis of EAFP2 indicates that (i) its dual activity is dependent on its chitin-binding properties and (ii) its amphipathic surface renders it active against the non-chitin-containing fungi (Huang et al. 2004). These peptides, compared with hevein, are three to five times more active against fungi and are strongly negated by calcium ions. Another antifungal peptide of this group, Ee-CBP, present in the bark and leaves of the spindle tree, *Euonymus europaeus*, has 10 cysteine residues comprising five disulfide bonds (Van den Bergh et al. 2002). The Ee-CBP precursor and a chitinase are synthesized as similar chimeric precursors consisting of an N-terminal hevein domain linked to a C-terminal chitinase-like domain by a hinge region (Van den Bergh et al. 2004). However, Ee-CBP undergoes cleavage of the N-terminal hevein domain, resulting in the formation of the final Ee-CBP molecule. Ee-CBP and the chitinase are active alone and act synergistically as antifungals against a number of phytopathogenic fungi (Van den Bergh et al. 2004).

**Knottins**

The knottin family of peptides include Mj-AMP$_1$ and Mj-AMP$_2$, which are present in *Mirabilis jalapa* seeds. They have 37 and 36 amino acids, respectively, and three disulfide bridges; they differ by only four amino acids (Cammue et al. 1992). Both inhibit the growth of phytopathogenic fungi, such as *B. cinerea*, *Colletotrichum lindenmuthianum*, and *Venturia inaequalis*. Mj-AMP$_1$ and Mj-AMP$_2$ have been expressed transgenically in tobacco but none of these plants show enhanced resistance to infection by *B. cinerea* or *Alternaria longipes*. The knottin, PAFP-s, produced by *Phytolaccace americana*, has significant sequence similarities and the same cysteine motif as the Mj-AMP peptides (Shao et al. 1999). PAFP-s is highly basic, consists of 38 residues with six cysteines comprising three disulfide bridges, and inhibits the growth of *F. oxysporum* and *Trichoderma viridae* (Shao et al. 1999). Several hydrophilic and positively charged residues surround the hydrophobic surface of PAFP-s, giving the molecule an amphiphilic character that is believed to be the main structural basis of its specificity to the fungal cell (Gao et al. 2001).

**Impatiens antimicrobial peptides**

*Impatiens balsamina* plant seeds contain a group of antifungal peptides (Ib-AMP$_1$ through Ib-AMP$_4$) that comprise about 0.5% of the total protein in the mature seed (Tailor et al. 1997). They are short, basic peptides, 20 amino acids in length that contain four cysteine residues forming two intramolecular disulfide bonds, have no significant homology with any peptides or proteins, and are nontoxic to human, plant, and insect cells in vitro (Broekaert et al. 1997; Tailor et al. 1997). The Ib-AMP family may include a β-turn but does not show evidence for either helical or β-sheet structure (Patel et al. 1998). They inhibit up to 50% of the growth of phytopathogens, such as *F. culmorum* and *Alternaria longipes* at $\leq$ 25 µg/mL (Tailor et al. 1997). Ib-AMP$_3$ is lethal to the germinating conidia of *Aspergillus flavus* and *F. moniliforme* at $\leq$ 25 µmol/L (De Lucca et al. 1999). Their mode of action is unknown, though it is known that at even high concentrations (500 µg/mL) no visible cell lysis occurs (Tailor et al. 1997).

**Cyclopeptide alkaloids**

Cyclopeptide alkaloids are produced by members of *Rhamnaceae* and other plant families (Panday and Devi 1990; Digel et al. 1983; Gournelis et al. 1997). Frangufoline, amphiheine H, rugosanines A and B, and nummularines B, K, R, and S are cyclopeptides whose antifungal properties have significant growth inhibition activity against *Aspergillus niger* but not *Candida albicans*, as shown by zonal inhibition studies (Panday and Devi 1990). Though frangufoline is known to bind to calmodulin (Han 1993), a Ca$^{2+}$-binding protein that mediates calcium-driven metabolic reactions, the mode of action of these molecules is unknown.

**Other peptides**

Other plant peptides include the 5-kDa pseudothionin-St1 present in potato tubers and found active against *F. solani* (Moreno et al. 1994). Another potato-produced antifungal peptide is sarkin-1, a 63 amino acid residue, cysteine-rich molecule (Segura et al. 1999). Sarkin-1 is constitutive and active at less than 10 µmol/L against potato fungal patho-
gens (Segura et al. 1999). Maize (Zea mays) seeds produce the antifungal peptides MBP-1 and an unnamed 22-kDa peptide (Duvick et al. 1992; Huynh et al. 1992). MBP-1 contains 33 amino acids with no free cysteines and is mostly a-helical. It shows no homology with thionins, though it is antifungal. MBP-1 inhibits the hylphal elongation of several phytopathogenic fungi, such as F. moniliforme and Fusarium graminearum, while the 22-kDa protein inhibits F. oxysporum and F. solani growth.

The intercellular washings of sugar beet leaves contain IWF6, which has no homology to any known antifungal protein. However, it has some homology, less than 26%, with agelenin, a neurotoxin from the venom of the spider Agelena opulentia that acts as a calcium blocker (Kristensen et al. 2000a). Though the IWF6 mode of action is unknown, this homology suggests that IWF6 may act in a similar manner (Kristensen et al. 2000a). IWF6 is a 37 amino acid peptide with six cysteines and has an isoelectric point of 7. As with the other peptides (e.g., AX1, AX2, IWF1–IWF4) isolated from the intercellular washings of sugar beet leaves, IWF6 is active against C. beticola (Kristensen et al. 2000a).

**Plant antifungal proteins**

The first response to infection by the plant is necrosis around the site of infection, the purpose of which is to reduce the spread of the disease (Matthews 1991). The hypersensitive response (HR) is a common response of plants to invasion by a plant pathogen. The HR is characterized by localized and rapid cell death (necrosis) and is restricted to tissue immediately surrounding the site of pathogen invasion (Oku 1994; Agrios 1997; Walton 1997). The HR is thought to be responsible for limiting the growth of the pathogen and, in this way, provides the host plant with a mechanism to resist further infection beyond the point of initial invasion. Typically, the HR develops within 24 h of infection, whereas cell death due to the development of disease symptoms takes several days. An effective HR may not always be visible when a plant remains resistant to attack by a pathogen, since it is possible for the HR to involve only a very few cells and remain unnoticed. The HR-induced resistance has been described in numerous diseases involving obligate parasites (fungi, viruses, mollicutes, and nematodes) as well as non-obligate parasitic fungi and bacteria (Agrios 1997). The HR is the culmination of plant defense responses initiated by the recognition of a pathogen-produced elicitor. This response is followed within hours or a few days by induction of a long-lasting, broad-based resistance and is considered as a very strong defense response induced in the plants by the pathogen itself (Stintzi et al. 1993; Shah and Klessig 1996; Caruso et al. 2001). The long-lasting response, termed systemic-acquired resistance, occurs in both the immediate area of infection as well as in the uninfected plant areas (Chester 1933; Ross 1961).

**Pathogen-related proteins**

An example of systemic-acquired resistance in plants is the production of pathogen-related (PR) proteins that were first reported in the 1970s (Gianinazzi et al. 1970; Van Loon and Van Kammen 1970). The term PR protein is defined as any protein genetically coded for by the host plant that is induced specifically in pathological or related situations following infection by a pathogen (Van Loon et al. 1994; Van Loon and van Strien 1999; Walton 1997). PR proteins are grouped into families (PR1–PR17) based on primary structure, serological relatedness, enzymatic activities, and biological activities (Christensen et al. 2002; Linthorst 1991; Stintzi et al. 1993; Van Loon et al. 1994; Van Loon and Van Strien 1999). Although PRs are most commonly observed during HRs of plants to microbial pathogens and appear to contribute to systemic-acquired resistance, the definition of the PR group does not require a role in resistance (Van Loon and van Strien 1999). PR proteins (see Table 2) also share common features related to structure, regulation of gene expression, and antifungal activity (Yun et al. 1997). They are stable in low pH, are resistant to protease activity, are monomers of low molecular weight, and show apoplastic location (Cheong et al. 1997). The stability in low pH and resistance to proteases are characteristics of food allergens. In fact, seven of the PR groups contain proteins with allergenic properties, with six of the groups containing food allergens (Ebner et al. 2001; Hoffmann-Sommergruber 2002).

PR proteins are widely distributed in healthy plants in trace amounts but are produced in much higher concentrations following elicitation caused by infection. They have typical physicochemical properties that enable them to resist acidic pH and proteolytic cleavage, allowing them to survive the harsh environments of the vascular compartment or cell wall or intracellular spaces (Stintzi et al. 1993). The significance of PR proteins lies in their strong antifungal and antimicrobial activity. Some inhibit conidial release and germination, while others are associated with strengthening the host cell wall and papillae. Certain PR proteins, such as β-1,3-glucanase (PR-2) and chitinases (PR-3, -4, -8, and -11), diffuse toward the invading pathogen and break down chitin-supported cell wall in certain fungi.

Most PR groups can be subdivided into two classes. Generally, class I proteins are localized in the vacuole of the plant cell, whereas class II proteins are extracellular. The class I and II proteins are related both structurally and immunologically but differ in their induction patterns (Brederole et al. 1991; Ward et al. 1991). The PR groups listed below are those reported to have antifungal properties.

**PR-1 proteins**

Members (PR-1a, PR-1b, and PR-1c) of the PR-1 group were first isolated from tobacco leaves after infection by the tobacco mosaic virus (Van Loon and Van Kammen 1970; Gianinazzi et al. 1970). However, they are now known to be present in rice, wheat, maize, barley, tobacco, and many other plants (Agrawal et al. 2000; Bryngelsson et al. 1994; Molina et al. 1999; Muradov et al. 1993; Rauscher et al. 1999). PR-1 family members are also present in non-plant systems. A novel PR-1 family member localizes to the cytosolic site of the endomembrane system in mammalian cells (Eberle et al. 2002). PR-1 proteins are characterized by their acidic or basic nature, their resistance to proteases, and their extracellular secretion (Bol and Linthorst 1990; Linthorst 1991; Van Loon 1985). Their mode of action is yet unknown (Van Loon and van Strien 1999). A protein, P14, was isolated from tomato leaves and determined to be similar in structure to the PR-1 group from tobacco (Camacho-Henríquez and Sanger 1982, 1984; Granell et al. 1987).
Examples of plant antifungal protein families.

<table>
<thead>
<tr>
<th>Protein (protein family)</th>
<th>Source</th>
<th>Mode of action</th>
<th>Target organism</th>
<th>In vitro MIC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum PR-1b (PR-1)</td>
<td>Unknown</td>
<td></td>
<td>Phytophthora infestans</td>
<td>100.0 µg/mL (IC90)</td>
<td>Niderman et al. 1995</td>
</tr>
<tr>
<td>Pr-32 (PR-2)</td>
<td>β-1,3-Glucanase</td>
<td>Fusarium solani</td>
<td>1.0 µg/well</td>
<td>Sela-Buurlage et al. 1993</td>
<td></td>
</tr>
<tr>
<td>Phasein (PR-3)</td>
<td>Chitinase</td>
<td>Fusarium oxysporum</td>
<td>µg/mL</td>
<td>Ye and Ng 2002</td>
<td></td>
</tr>
<tr>
<td>CBP-20 (PR-4)</td>
<td>Chitinase</td>
<td>Trichoderma viridae</td>
<td>6.7 µg/mL</td>
<td>Ponstein et al. 1994</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lysis</td>
<td></td>
<td></td>
<td>0.5 µg/mL</td>
<td>Roberts and Selitrennikoff 1991</td>
</tr>
<tr>
<td>Oxalis tuberosa Ocatin (PR-10)</td>
<td>Inhibits hyphal extension</td>
<td>Fusarium oxysporum</td>
<td>µg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco chitinase (PR-11)</td>
<td>(tobacco) Endochitinase</td>
<td>Alternaria radicina</td>
<td>5–10 µg/well</td>
<td>Melchers et al. 1994</td>
<td></td>
</tr>
<tr>
<td>Hv-Ti3 (thionin)</td>
<td>Trypsin inhibitor; inhibits hyphal growth</td>
<td>90.0 µg/mL (IC50)</td>
<td>Fusarium culmorum</td>
<td>Terras et al. 1993</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribosome-inactivating protein; inhibits protein synthesis</td>
<td></td>
<td></td>
<td>Parkash et al. 2002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Permeabilize membranes</td>
<td>Verticillium dahliae</td>
<td>45.0 µg/mL (IC50)</td>
<td>Terras et al. 1993</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibits conidial germination</td>
<td>Barytia cichorae</td>
<td>40.0 µg/mL (IC50)</td>
<td>Broekaert et al. 1989</td>
<td></td>
</tr>
<tr>
<td>Note: MIC, minimum inhibitory concentration; IC50, concentration producing 50% growth inhibition.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PR-2 proteins**

The PR-2 proteins (β-1,3-glucanases) have been grouped into three classes on the basis of amino acid sequence (Beffa and Meins 1996; Cote et al. 1991; Leal et al. 1991; Linthorst 1991; Meins et al. 1992; Nielsen et al. 1997; Payne et al. 1990). PR-2, class I, glucanases are basic proteins of approximately 34 kDa and are present in plant vacuoles. Five PR-2-type proteins are produced by rice in response to infection by Rhizoctonia (Bera and Purkyayastha 1997). The interstitial tissues of barley leaves contain Pr-32, an antifungal 32-kDa member of the PR-2 protein group (Zareie et al. 2002). Though active alone, Pr-32 is more active in the presence of different combinations of a chitinase and three thaumatin-like proteins also present in the leaf extracts. An extracellular, acidic, 32-kDa PR-2 protein is produced in cucumber leaves upon infection by Colletotrichum lagenarium (Ji and Kuc 1995). A time-course study shows that the increase in β-1,3-glucanase production is associated with induced resistance against the fungus (Ji and Kuc 1995).

**PR-3 proteins**

Plants are known to produce chitinases (PR-3 proteins) that are co-ordinately induced with glucanases (PR-2 proteins) as part of the HR to pathogen infection (Meins and Ahl 1989; Vogeli et al. 1988). PR-3 chitinases have molecular masses between 26 and 43 kDa (Nielsen et al. 1997; Watanabe et al. 1999) and act by cleaving the chitin in the fungal cell wall, thus rendering the cells osmotically sensitive. Plants known to produce PR-3 proteins include bean (Boller et al. 1983), wheat (Molano et al. 1979), and tobacco (Shinshi et al. 1987). Two chitinases, Phaseins A and B, having molecular masses of 28 and 32 kDa, respectively, are present in Phaseolus vulgaris (Ye and Ng 2002a). Phasein A demonstrates potent antifungal activity toward F. oxysporum and Phytophthora piricola, whereas Phasein B is more active against P. piricola than F. oxysporum (Ye and Ng 2002a). Antifungal chitinases have also been isolated from garlic (Van Damme et al. 1993) and leek (Vergauwen et al. 1998).

**PR-4 proteins**

PR-4 proteins bind chitin and have molecular masses much smaller (13–14.5 kDa) than those of the PR-3 proteins and are classified into two groups (Friedrich et al. 1991; Hejgaard et al. 1992; Ponstein et al. 1994; Van Damme et al. 1999). The class I group has amino acid sequences similar to hevein (Friedrich et al. 1991; Koo et al. 1998; Van Damme et al. 1999), while the class II proteins lack a chitin-binding domain (Selitrennikoff 2001). PR-4 proteins have been isolated from many plants, including maize, potato, tobacco, barley, and tomato (Bravo et al. 2003; Friedrich et al. 1991; Hejgaard et al. 1992; Koo et al. 1998; Van Damme et al. 1999). Class I PR-4 proteins bind to β-chitin in developing fungal cell wall, resulting in inhibition of growth; however,
the mechanism of action of the class II PR-4 proteins is not known (Borman et al. 1999; Selitrennikoff 2001). One example of a PR-4 protein is CBP20, which is 20 kDa in size (Ponstein et al. 1994) and is induced by pathogens or wounds in tobacco (Nicotiana tabacum) leaves. It is active in vitro against T. viridae and F. solani by causing lysis of the germ tube and (or) growth inhibition. CBP20 also acts synergistically with a tobacco class I chitinase against F. solani and with a β-1,3-glucanase against F. solani and Alternaria radicina (Ye and Ng 2002a). Maize produces a class II chitinase, ZmPR4, which is accumulated in plant cells that first establish contact with the pathogen (Bravo et al. 2003). ZmPR4 production is induced by wounding of the plant and by several compounds, such as abscisic acid, methyl jasmonate, and moniliformin (a mycotoxin produced by the fungus F. moniliforme, a maize pathogen).

PR-5 proteins

The PR-5 proteins are considered thaumatin-like proteins (TLP) because their amino acid sequence is highly homologous to that of thaumatin, an intensely sweet protein from Thaumatococcus danielli (Cornelissen et al. 1986; Richardson et al. 1987). The majority of these proteins are approximately 22 kDa and are stabilized by eight disulfide bonds (Selitrennikoff 2001). This structure allows the PR-5 proteins to be resistant to protease degradation (Roberts and Selitrennikoff 1990). PR-5 proteins can be categorized into three subclasses based on their isoelectric point, as well as their acidic, neutral, and basic properties (Koiwa et al. 1994). In vitro, the PR-5 proteins inhibit hyphal growth and mediate hyphal and spore lysis (Hejgaard et al. 1991; Roberts and Selitrennikoff 1990; Vigers et al. 1991, 1992; Woloshuk et al. 1991), which is correlated with plasma membrane permeabilization and dissipation of the plasma membrane potential (Abad et al. 1996). The primary role of PR-5 proteins in planta is not known, but current models suggest that they form lethal transmembrane pores in fungi (Skadsen et al. 2000). A PR-5 protein from pumpkin, PLPT, rapidly hydrolyzes the hyphal tips of Neurospora crassa and significantly inhibits growth of F. oxysporum (Cheong et al. 1997). Barley seeds contain the antifungal proteins R and S, which are homologous to thaumatin and other PR-5 family proteins (Hejgaard et al. 1991). These proteins, in vitro, inhibit the growth of C. albicans and T. viridae and act synergistically with barley grain chitinase C (Hejgaard et al. 1991). Tobacco cells produce PR-5d, a hydrophobic, neutral protein that inhibits phytopathogenic and nonphytopathogenic fungi (Koiwa et al. 1997). Another PR-5 protein present in tobacco is osmotin (Singh et al. 1987), which is active against P. infestans, C. albicans, N. crassa, and Trichoderma reesei (Woloshuk et al. 1991; Vigers et al. 1992). Osmotin is a basic PR-5 protein that interacts with cell surface phosphomannan, suggesting that fungal cell surface polysaccharides control target specificity of plant PR-5 proteins (Ibeas et al. 2000). Overexpression of a heterologous cell wall glycoprotein in F. oxysporum increases virulence and resistance to osmotin, which indicates that the fungal cell wall components can increase resistance to plant defense proteins and affect virulence (Narasimhan et al. 2003). Zeamatin is a 22-kDa PR-5 protein present in Z. mays seeds (Roberts and Selitrennikoff 1990). Zeamatin causes the rapid release of cytoplasmic fluid and inhibits the growth of C. albicans, N. crassa, and T. reesei (Roberts and Selitrennikoff 1990; Vigers et al. 1991). Proteins homologous to zeamatin are present in barley (Hejgaard et al. 1991) and oat (Vigers et al. 1991). Antizeamatin serum cross reacts with proteins of similar size from a wide range of cereals, which suggests that these proteins are ubiquitous and structurally related (Vigers et al. 1992).

PR-10 proteins

PR-10 proteins are a family of small homologous, primarily acidic molecules present in a variety of angiosperms, monocots, and dicots (Ostmark et al. 1998). Several members of this group possess ribonuclease properties and are antifungal. One such protein is CaPR-10 that is present in hot pepper (Capsicum annuum). It is an intracellular protein (17.3 kDa) located in the cytosol and is known to inhibit hyphal extension of Phytophthora capsici, possibly by inhibition of translation activity (Lam and Ng 2001; Park et al. 2004). PR-10 proteins share similarity with the allergen Bet v 1, known to have ribonuclease activity in white birch and in major latex proteins found in aradiopsis, bell pepper, melon, strawberry, and tobacco (Bufo et al. 1996; Ostmark et al. 1998). A group of inducible, small molecular weight proteins with acidic natures and N-terminal amino acid sequences similar to the PR-10 proteins are present in leaves from Lupinus albus (Pinto and Riccardo 1995). These proteins, PR-p16.5, PR-p16.5b, and PR-p16.5c are induced upon infection with Colletotrichum gloeosporioides.

Ocatin is a 18-kDa storage protein in the Andean tuber, oca (Oxalis tuberosa), and is classified as an antifungal of the PR-10 group. This protein constitutes between 40% and 60% of the total soluble oca tuber proteins and, in vitro, inhibits the growth of several phytopathogenic fungi, including F. oxysporum and Rhizoctonia solani (Flores et al. 2002). The concentration of ocatin is highest at harvest time and decreases during storage. The amino acid sequence of ocatin is very similar to proteins of the Bet v 1/PR-10 families and major latex proteins (Ostmark et al. 1998).

Other antifungal PR proteins

Cucumber plants (Cucumis sativus) produces a chitinase found in both leaves infected with Colletotrichum lagenarium and in uninfected leaves up to five leaves above the infected one (Métraux et al. 1988). This chitinase has a molecular mass of 28 kDa and is classified as an example of PR-8 proteins. Tobacco plants produce a number PR proteins, among them are chitinases belonging to the PR-11 group (Melchers et al. 1994). These two chitinase groups are related and have molecular masses of 41 and 43 kDa. Both have endochitinase activity toward T. viridae and Alternaria radicina and show synergy with a tobacco β-1,3-glucanase against F. solani germings (Melchers et al. 1994). Barley produces a germin-like oxalate oxidase, classified as a PR-15 protein, which produces hydrogen peroxide in response to infection by the causative agent of powdery mildew, Erysiphe graminis (Hang et al. 1995; Zhang et al. 1995). The oxalate oxidase appears 1–3 days earlier than does a PR-1 protein.

The PR-17 group contains two protein families, WCI-5 from wheat and HvPR-17 and HvPR-17b from barley, with antifungal properties (Christensen et al. 2002; Görlich et al. 2005). © 2005 NRC Canada
The two PR-17 proteins from barley are extracellular and may affect cell wall metabolism or signal transduction and are active against the fungus *Blumeria graminis*, which causes powdery mildew disease on barley fungus. WCI-5 in wheat is an induced lipoxygenase active against *Erystipe graminis*.

**Trypsin inhibitors**

Corn genotypes resistant to *Aspergillus flavus* express high levels of a 14-kDa trypsin inhibitor (Brown et al. 1999; Chen et al. 1998, 1999a, 1999b), which affects conidial germination, hyphal extension, and fungal α-amylases. Banks et al. (2002) showed this trypsin inhibitor can cross the blood–brain barrier in mice, indicating a possible role for this protein in the treatment of fungal infections in the central nervous system of mammals. Barley leaves also produce a trypsin inhibitor that in vitro, enhances (2- to 55-fold) the antifungal activity of wheat and barley thionins (Terras et al. 1993b), which suggests that a synergistic effect occurs in the plant when fungal infection occurs. Recently, a new trypsin–chymotrypsin inhibitor was found present in broad bean (*Vicia faba*) seeds (Ye and Ng 2002b). This protein, with a molecular mass of 13 kDa, not only exhibits antifungal properties against *Mycosphaerella arachidicola* and *P. piricola* but also inhibits the activity of human immunodeficiency virus-1 reverse transcriptase.

**Ribosomes-inactivating proteins**

Ribosome-inactivating proteins (RIPs) are widely distributed among higher plants (Mehta and Boston 1998) and inhibit protein synthesis as a result of their N-glycosidase activity (Endo and Tsurugi 1988). These proteins inactivate ribosomes by cleaving a single adenine from the large rRNA, thus arresting protein synthesis at the translation step (Barbieri et al. 1993; Sharma et al. 2004). It is possible that as little as one RIP molecule per cell is capable of turning off protein synthesis (Strirpe and Barbieri 1986). In vivo, RIPs may act synergistically with chitinases and β-1,3-glucanases against invasive fungi (Broekaert et al. 1989; Kombrick et al. 1988; Mauch et al. 1988). RIPs are composed of several categories: single-chained type 1 RIPs, double-chained type 2 RIPs, and small RIPs. The types 1 and 2 are 30 and 60 kDa, respectively, while the small RIPs are approximately 10 kDa. Type 2 RIPs have two subunits, an “A” (Gal/GalNAc-binding subunit) chain and a “B” chain having lectin properties. Ricin, an extremely toxic molecule composed of several categories: single-chained type 1 RIPs, double-chained type 2 RIPs, and small RIPs. The types 1 and 2 are 30 and 60 kDa, respectively, while the small RIPs are approximately 10 kDa. Type 2 RIPs have two subunits, an “A” (Gal/GalNAc-binding subunit) chain and a “B” chain having lectin properties. Ricin, an extremely toxic molecule composed of several categories: single-chained type 1 RIPs, double-chained type 2 RIPs, and small RIPs.

**Lectins**

Lectins are proteins or glycoproteins that recognize and bind reversibly to carbohydrate moieties of complex glycoconjugates, inhibit fungal conidial germination, alter germ tubes, and inhibit hyphal growth (Allen et al. 1973; Lotan and Sharon 1973; Mirelman et al. 1975). Plants are known to produce lectins that protect the plant from fungal infection (Keen 1992). They produce a wide variety of lectins that either specifically bind to oligomers of N-acetylglucosamine and (or) chitin (Van den Bergh 2004). The great majority of them belong to the ubiquitous family of chitin-binding proteins composed of hevein domains (Van den Bergh 2004). For example, wheat germ agglutinin (WGA) lectin, present in wheat germ, has an affinity for N-acetylglucosamine and binds to chitin-containing fungal walls (Ciopraga et al. 1999). WGA binds to the chitin in the fungal cell wall thus disrupting the steady state between hydrolysis and synthesis of wall polymers (Ciopraga et al. 1999). This binding of WGA to the fungal cell wall polymers affects the inner compartment, as observed in fungal morphological changes, namely the swelling and vacuolation of cellular content and lysis of hyphal tips (Ciopraga et al. 1999).

The stinging nettle, *Urtica dioica*, produces UDA (*Urtica dioica* agglutinin), a small, single-chain (8.5 kDa) fungistatic lectin that is unusually heat and acid resistant and is very similar to the amino acid sequence of WGA (Broekaert et al. 1989). UDA, which consists of two cysteine-rich chitin-binding domains (Beintema and Peumans 1992), inhibits the hyphal growth of several phytopathogenic fungi and is three to five times more active against fungi than hevein (Broekaert et al. 1989; Van Parijs et al. 1991). The growth-inhibiting effect of UGA appears to be based on a specific phase of fungal growth and is temporal, suggesting that the fungi...
have an adaptation mechanism (Dies et al. 1999). UGA, though similar in amino acid homology to hevein, agglutinates red blood cells whereas hevein does not (Van Parijs et al. 1991). These antifungals also differ in that the C terminal of hevein shows homology to chitinases, whereas the C terminal of UGA shows homology to tobacco PR-4 chitinases (Broekaert et al. 1990; Linthorst et al. 1991).

The lectin STA (Solanum tuberosum agglutinin) is a hydroxyproline-rich glycoprotein (HRGP) present in potato tubers that occurs predominantly at the cell surface or as soluble mucin-like molecules (Kieliszewski et al. 1994). STA is the best characterized HRGP lectin, which binds to chitin and is composed of two dimers. However, recent studies show that STA is composed of two homologous modules of twin hevein domains interspersed by an extensin-like domain of approximately 60 amino acid residues that is hydroxyproline-rich (Van Damme et al. 1993). It irreversibly inhibits conidial germination of F. oxysporum by preventing conidial germination and lysis of the germ tube (Gozia et al. 1993).

Gastrodianins (Wang et al. 2001) are a family of mannose-binding, antifungal proteins produced by Nicotiana tabacum (tobacco) and the orchid Gastrodia elata, a traditional Chinese medicinal herb (Wang et al. 2001). Fungal infection induces the orchid to produce the gastrodianin antifungal proteins (GAFPs), a group of mannose-binding lectins at high levels in the cortex of the terminal corms (Hu and Huang 1994). At least five GAFPs (GA-1 through GA-5) have been identified based on their cDNA sequences (Hu et al. 1999; Wang et al. 1999, 2001). GA-1 is a monomer with a molecular mass of 10 kDa and inhibits the growth of a number of phytopathogenic fungi, including B. cinerea, Rhizoctonia solani, and Gibberella zeae, by inhibiting conidial germination and hynphal elongation (Xu et al. 1998). Besides mannose, GA-1 binds with chitin and N-acetylglucosamine and has an N-terminal sequence that is homologous to other known lectins of the Orchidaceae (Xu et al. 1998).

**Other antifungal proteins**

Other antifungal proteins include allivin, a novel 13-kDa protein from garlic, with N-terminal sequencing having very little similarity to the chitinases present in garlic and leek (Van Damme et al. 1993; Vergauwen et al. 1998). A novel deoxyribonuclease with antifungal activity is present in Asparagus officinalis seeds (Wang and Ng 2001a). It is a 30-kDa molecule with activity against B. cinerea. A 36-kDa antifungal protein is present in the edible chive Allium tuberosum (Lam et al. 2000) with an N-terminal sequence similar to chitinases but lacking the cysteine residues characteristic of a cysteine-rich domain present in chitinases of other Allium species. It is active against R. solani, F. oxysporum, and B. cinerea. A similar chitinase-like antifungal protein is present in Panax notoginseng roots (Lam and Ng 2001).

**Conclusion**

The need for novel, potent, antifungals increases as fungi develop resistance to currently employed antifungals in agriculture and medicine. The search for new antifungals has also developed because of the increased frequency of invasive mycoses, particularly in immunocompromised patients, by soil or plant fungal pathogens (e.g., Alternaria, Curvularia, and Rhizopus) formerly considered as fungi of low virulence but now considered as emerging pathogens. Plants have been defending themselves against fungal infection for millions of years, so it is not surprising that they produce a wide range of effective, antifungal compounds. Plants offer an enormous source of potent, novel antifungal compounds with potential utility in agriculture, medicine, food processing, and other areas.

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