Small cysteine-rich proteins from plants: a rich resource of antimicrobial agents

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Significant research effort has focussed in recent years on identifying natural and environmentally friendly sources of antimicrobial agents, for applications in agriculture as well as human health. This quest has led to identification of a number of small, cysteine-rich, and typically basic proteins which have antibacterial and antifungal activities. These include the non-specific lipid transfer proteins (ns-LTPs), puroindolines (PINs), and thionins. Extensive biochemical studies have shown higher order structures as well as specific residues and/or domains to be important for membrane activities. Numerous in-vitro tests have confirmed their antibacterial and/or antifungal activities, some studies showing variations in their growth inhibitory concentrations or strain specificities. Transgenic transfers of the respective genes into diverse backgrounds have also led to successful in-planta expression and enhanced microbial defence. The ns-LTPs, PINs and thionins thus make excellent candidates as biocontrol agents of plant pathogens.

Keywords: Non-specific lipid transfer proteins, puroindolines, thionins, antimicrobial peptides.

1. Introduction

Significant research effort has occurred in the last 20 years or so on identifying new-generation biocidal agents for human health or plant protection applications. The main expectations of this focus are that the chances of the host species developing resistance to these agents would be minimal, and that these will also be more environmentally friendly compared to chemical agents for pathogen control. A number of antimicrobial proteins and peptides (AMPs) have been reported in the course of this work, particularly from the seeds and leaves of various plants. For example, the interest in wheat and barley seed lipid-binding proteins was primarily due to the association of lipid functionality to food technological properties, e.g., association with bread loaf volumes or stability of beer foam. However, a number of wheat and barley lipid-binding proteins were also found to be antimicrobial in nature. The purothionins were amongst the first seed proteins noted to exhibit bactericidal and fungicidal properties in vitro (Fernandez de Caleya et al., 1972; Blochet et al., 1993). Other AMPs from wheat and/or barley include albumins, the structurally related non-specific lipid binding proteins (ns-LTPs) (Marion et al., 1994) and puroindolines (PINs) (Gautier et al., 1994), and defensins (reviewed in Garcia-Olmedo et al., 1998). The ns-LTPs, PINs as well as other AMPs are suggested to protect the seed (Gautier et al., 1994), possibly from soil-borne pathogens, and many of these genes are found to be up-regulated during seed development (McIntosh et al., 2007). It thus appears that plant seeds are a rich source of AMPs. The biochemical properties of ns-LTPs, PINs and thionins and their potential as biocontrol agents are discussed below.

2. Lipid-Transfer Proteins

2.1 Biochemical properties of ns-LTPs:

The non-specific lipid transfer proteins (ns-LTPs) are small, basic proteins that are ubiquitous in, but restricted to, higher plants, and suggested to be mainly involved in inter-membrane lipid trafficking at cellular and extracellular levels. The term non-specific refers due to their proposed ability to mobilize different types of polar lipids, phospholipids and glycolipids, as well as hydrophobic monomers of cutin and suberin (e.g., fatty acid chains, fatty alcohols, phenolic and hydroxy-fatty acids) that make up the external protective layers of plants (Kader 1996; Garcia-Olmedo et al., 1998; Douliez et al., 2002). The ns-LTPs typically range in size from 6.5-10.5 kilo Dalton (kDa) (Douliez et al., 2000), although some members of the wheat ns-LTP superfamily reach 13-15 kDa due to N- or C-terminal extensions of mature proteins (Boutrot et al. 2008). They are typically basic (pI 8.5-12), and nearly all members have a characteristic 8-cysteine (Cys) motif (8 CM) backbone in the pattern Cys1-Xn-Cys2-Xn-Cys3Cys4-Xn-Cys5XCys6-Xn-Cys7-Xn-Cys8 that form four disulphide bridges (Kader 1996; Douliez et al., 2000; Boutrot et al., 2008). However, variations in the number of Cys have been noted in some members of the Arabidopsis, rice and wheat ns-LTP families (Boutrot et al., 2008). The N-terminal sequences of the 9kDa proteins show a high homology, including between dicots and monocots, and complete conservation of a specific Val, near-complete conservation of certain Gly, Ser and Pro residues, and conservation of hydrophobic residues at specific sites (Rogozhin et al., 2009).
Almost all ns-LTPs lack tryptophan (Trp) residues except for a few isoforms in Arabidopsis and rice that have 1-2 Trp (Boutrot et al., 2008). The ns-LTPs were traditionally classified into two main subclasses for a number of years into ns-LTP1 (typically 7 kDa) and ns-LTP2 (typically 9 kDa). The advent of bioinformatics analyses of genomes and transcriptomes has now led to identification of very large gene families, e.g., in wheat (Boutrot et al., 2008), and much more thorough comparisons of the organisation of the Cys backbone and the conserved/nonconserved sites. Thus the ns-LTPs have been classified into five groups (Jang et al., 2007), or nine subfamilies based on the spacing between the Cys as well as certain residues that distinguish some of the groups (Boutrot et al., 2008).

The ns-LTP1 (Ginec et al., 1994) shows a polypeptide backbone that exhibits a secondary structure of a bundle of four α-helices separated by flexible loops, the tertiary structure held together by four disulphide bridges between the eight conserved Cys. The helical structure is considered important for lipid binding, with a hydrophobic cleft formed in the C-terminal half comprising the lipid-binding site. The weak adsorption of these proteins at the lipid bilayer interface and the lipid-binding cleft possibly allow them to mediate inter-membrane lipid transfers. The ns-LTP2 (Douliez et al., 2001) also shows a similar structure despite differences in length compared to ns-LTP1. The structure comprised of four α-helices that make up a scaffold that forms a hydrophobic cavity has been confirmed through solution structures of the barley orthologue LTP1 in its un-liganded (Heinemann et al., 1996) and liganded forms (Lerche and Poulsen, 1998), and crystal structure for the well-known peach ns-LTP (allergen Pru p 3) (Pasquato et al., 2006). Curiously, the distantly related anion lipid binding protein Ace-AMP1 (not considered a typical ns-LTP) lacks the cavity required for lipid binding for transfers, and has a C-terminal cleavable peptide that shows characteristics of vacuole-targeted proteins, however, the protein appears to be able to bind to lipid bilayers and alter membrane permeability, essential for antimicrobial activities (Cammue et al., 1995; Tassin et al., 1998). The antimicrobial properties of ns-LTPs have been associated with an ability of membrane interaction (Kader 1996; Regente et al., 2005).

A collateral effect of this structure appears to be the allergenicity of some of the ns-LTPs, the peach LTP1 being a major allergen that can cause severe reactions including anaphylaxis. Further, recent studies involving proteolysis at very low pH (to simulate the gastric proteolysis) and pH 6.5 (to simulate intestinal proteolysis) on purified peach LTP as well as barley LTP1 in its liganded and unliganded form showed their structures were rigid and highly resistant to proteolysis, despite having a number of predicted peptic and tryptic proteolysis sites, and confirmed that the indigestibility of these proteins was most likely responsible for their strong allergenic potential (Wijesinha-Bettoni et al., 2010).

2.2 Antimicrobial activities of ns-LTPs:

The ns-LTPs are distributed in high concentrations over exposed surfaces and in the vascular system, and are proposed to be involved in membrane biogenesis and secretion of protective materials, as well as numerous other functions during normal development as well as defence under infections and wounding (reviewed in Kader 1996; Marion et al., 2007). The roles in defence may involve direct antimicrobial properties, as well as indirect roles through secretion of defensive barriers of earlier-mentioned chemicals on the external layers.

A number of LTPs exhibit antibacterial or antifungal properties in vitro, hence have been classified as the class PR-14 of the pathogen-resistance proteins (Velazhahan et al., 2001). The first ns-LTP found to be antifungal was a highly basic (pl >10.5) dimeric protein from radish seeds, with 9kDa subunits, which was effective against a number of genera (Table 1), by preventing hyphal growth rather than spor germination (Terras et al., 1992). Proteins extracted from leaves of barley and maize showed activity against the fungal pathogens Clavibacter michiganensis subsp. sepedonicus, Fusarium solani and the also bacterial pathgen Pseudomonas solanacearum (Molina et al., 1993). The study led to important observations that these ns-LTPs were present in plant tissue at concentrations well above those required for complete inhibition of the pathogen in-vitro; that ns-LTPs were more active than thionins (see below) against bacterial strains compared to fungi, and that the extent of activity varied against different pathogens (Molina et al., 1993). The ns-LTP extracted from radish seeds showed activity against two fungal strains and only weak activity against a Gram-positive bacterium, while the Ace-AMP1 from onion seeds (see above) showed strong activity against all twelve fungal strains (of different genera/species) tested, but only against two Gram-positive bacteria and not Gram-negative bacteria, and the ns-LTPs from maize and wheat seeds lacked antifungal activity and zm-nsLTP was only weakly active against a Gram-positive bacterium (Cammue et al., 1995) (Table 1). The ns-LTP extracted from pear millet seeds also inhibits mycelial growth of two strains tested (Velazhahan et al., 2001). Transgenic work has also confirmed the causative roles of ns-LTPs and other lipid-transfer proteins in pathogen resistance. For example, transfer and expression of barley LTP in tobacco and Arabidopsis and challenging the transgenic plants by fungal pathogens showed decrease in total areas of lesions as well as diameter of individual lesions (Molina and Garcia-Olmedo, 1997). The onion Ace-AMP1 likewise imparts fungal resistance to transgenic geranium (Bi et al., 1999) and rose (Li et al., 2003).

Studies involving analysis of gene expression have also shown that a number of ns-LTP isoforms are differentially expressed under fungal or bacterial infections (reviewed in Garcia-Olmedo et al., 1995). Of two tandemly arranged barley genes, HvLtp4.2 and HvLtp4.3, expression of a reporter gene driven by the promoter of HvLtp4.3 was upregulated upon Xanthomonas campestris pv. Translucent infection, and down-regulated under Pseudomonas syringae pv. Japonica, while expression driven by HvLtp4.2 promoter did not vary (Molina et al., 1996). Promoter
analysis also showed the rice gene LTP1 has a role in organ development, and its expression is inducible only under attack by the fungal pathogen *Magnaporthe grisea* that causes rice blast (Guiderdoni et al., 2002). The ns-LTP from sunflower seeds, Ha-AP10 (similar to Arabidopsis LTP4), affected spore viability of *Fusarium solani*, had lethal effects at higher concentration, and was the first ns-LTP shown to permeabilise the membranes of intact spores (Regente et al., 2005). A novel 9 kDa ns-LTP from a wild grass, *Echinochloa crusgalli*, was shown to inhibit the growth of zoosporangia of two major phytopathogenic fungi that cause the late blight of potato and tomato and root rot of herbs, and was more potent than other ns-LTPs (Rogozhin et al., 2009) (Table 1). In summary, the effects of various ns-LTPs include inhibition of hyphal growth and/or spore germination, with or without effects on spore or cell morphology. The effective concentrations also vary for different proteins. Further, there may be some temperature dependence on activity; e.g., the ns-LTP from *Echinochloa crusgalli* appears to be more effective at lower temperature compared to room temperature (Rogozhin et al., 2009).

**Table 1: Antimicrobial properties of non-specific lipid transfer proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Active against</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley and maize leaves unnamed extracted proteins</td>
<td><em>Fusarium solani</em>, <em>Clavibacter michiganensis</em> subsp. <em>sepedonicus</em>, <em>Pseudomonas solanacearum</em>.</td>
<td>Molina et al., 1993</td>
</tr>
<tr>
<td>Radish seeds ns-LTP</td>
<td><em>Pyricularia oryzae</em>, <em>Verticillium dahliae</em>, <em>Bacillus megaterium</em></td>
<td>Cammue et al., 1995</td>
</tr>
<tr>
<td>Onion seeds Ace-AMP1</td>
<td><em>Alternaria brassicola</em>, <em>Ascochyta pisi</em>, <em>Botrytis cinerea</em>, <em>Colletotrichum lindemuthianum</em>, <em>Fusarium culmorum</em>, <em>Fusarium oxysporum f. sp. Pisi</em>, <em>Fusarium oxysporum f. sp. lycopersici</em>, <em>Nectria haematococca</em>, <em>Phoma betae</em>, <em>Pyrenopora tritici-repentis</em>, <em>Pyricularia oryzae</em>, <em>Verticillium dahliae</em>, <em>Bacillus megaterium</em>, <em>Sarcina lutea</em>.</td>
<td>Cammue et al., 1995</td>
</tr>
<tr>
<td>Transgenic tobacco and Arabidopsis expressing barley LTP2</td>
<td><em>Pseudomonas syringae</em>.</td>
<td>Molina and Garcia-Olmedo, 1997</td>
</tr>
<tr>
<td>Transgenic geranium expressing onion Ace-AMP1</td>
<td><em>Botrytis cinerea</em>.</td>
<td>Bi et al., 1999</td>
</tr>
<tr>
<td>Beet leaves IWF1/2</td>
<td><em>Abschnitt 1.01Cercospora beticola</em>.</td>
<td>Neilsen et al., 1996</td>
</tr>
<tr>
<td>Pearl millet seeds ns-LTP</td>
<td><em>Rhizoctonia solani</em>, <em>Trichoderma viride</em>.</td>
<td>Velazhahan et al., 2001</td>
</tr>
<tr>
<td>Transgenic rose expressing onion Ace-AMP1</td>
<td><em>Sphaerotheca pannosa</em>.</td>
<td>Li et al., 2003</td>
</tr>
<tr>
<td>Sunflower seeds Ha-AP10</td>
<td><em>Fusarium solani</em>.</td>
<td>Regente et al., 2005</td>
</tr>
<tr>
<td>Barnyard grass seeds Ec-LTP</td>
<td><em>Phytophthora infestans</em>; <em>Helminthosporium sativum</em>.</td>
<td>Rogozhin et al., 2009</td>
</tr>
<tr>
<td>Coffee (Robusta-type) seeds Cc-LTP1 (two isoforms)</td>
<td><em>Candida albicans</em>, <em>Candida tropicalis</em>.</td>
<td>Zottich et al., 2011</td>
</tr>
</tbody>
</table>

The above-summarised literature on the antimicrobial abilities of ns-LTPs thus supports the suggestion (Blein et al., 2002) that these proteins may be involved in signalling pathways in the host cell and/or the pathogen during infection. In this context, new research shows a LTP from Chinese cabbage, CaMBp10, was found to be a calmodulin-binding protein, regulated by phosphorylation in a calcium-dependent manner, at the C-terminal region that also has the CaM-binding domain (Li et al., 2011), thus providing insights into the regulation of this and perhaps other ns-LTPs. A recently isolated 9 kDa protein from coffee seeds, CcLTP1, is comprised of two isoforms and inhibits the growth of one species of the yeast *Candida* and alters the morphology of another, but is also unique in being the first LTP found to
inhibit the activity of human salivary α-amylase (Zottich et al., 2011). The implications or applications of this are not yet clear.

Further, a number of studies have also shown that specific ns-LTP isoforms are up-regulated under abiotic stresses (e.g., salt, drought, methyljasmonate, salicylate, abscisic acid, ethephon) (Garcia-Olmedo et al., 1995). The barley HvLtp4.2 and HvLtp4.3 (see above) showed up-regulation under cold treatment (Molina et al., 1996). The rice LTPI which was found to be inducible under Magnaporthe grisea infection (see above), was also inducible under wounding, the tissue distribution of expression indicating a role in strengthening the structural defences against mechanical damage in addition to pathogen challenge (Guiderdoni et al., 2002). The wheat TaLTPI was found to respond to dehydration and salt stresses, and its upstream 337 bp region showed a number of elements which are putative binding sites for the MYB and MYC transcription factors that regulate signal transduction pathways related to water stress (Jang et al., 2004). Some of the subgroups of wheat nsLTPs were also responsive to abiotic stresses, and promoter studies by reporter gene expression indicated most groups were strongly expressed during development, indicative of functional specialisations within the superfamily (Wang et al., 2010). Studies on fourteen ns-LTPs from the tree species Tamarix hispida (the genus contains species that are tolerant to diverse abiotic stresses) showed the genes are responsive to salt, dehydration, alkalinity and cadmium, and strongly up-regulated upon exposure to abscisic acid (Wang et al., 2009), indicative of roles in abiotic stress tolerance, in addition to the roles in pathogen response pathways mentioned above.

3. Puroindolines

3.1 Biochemical properties of PINs:

The wheat puroindolines, PINA and PINB, are 148 amino acids long (approximately 14kDa), basic (pI 10.5) and encoded by the Pina-D1 and Pinb-D1 genes, respectively (Gautier et al., 1994). Both PINs contain a backbone of ten highly conserved Cys, with a tertiary structure similar to that of ns-LTPs (Gautier et al., 1994; Marion et al., 1994). The biochemical properties of PINs and their effects on grain texture have been reviewed elsewhere (Bhave and Morris, 2008a). However, the effect on grain texture appears to be co-incidental than biologically meaningful. This is due to the fact that PINs share key tertiary structural properties with ns-LTPs, and thus, right from the time of their discovery, PINs have been suggested to be primarily membrano-toxins, with roles in seed and seedling defence against microbial pathogens (Blochet et al., 1993; Gautier et al., 1994). In this context, two structural properties of PINs are noteworthy. Firstly, they form a secondary structure very similar to that of ns-LTPs (see above), comprised of four α-helices separated by loops of variable lengths, with the tertiary structure held together by 5 disulphide bridges, four being identical to those in ns-LTPs and the 5th being due to the two additional Cys in PINs (Le Bihan et al., 1996; Kooijman et al., 1997). PINs are thus suggested to be members of a larger family that originated from a common ‘helicoidal’ ancestral protein and includes ns-LTPs and amylase and protease inhibitors (Le Bihan et al., 1996). The different members of the family have inhibitory activities against plant predators and pathogens, specific residues in the loops likely imparting different properties such as membrane binding or enzyme inhibition (Le Bihan et al., 1996). Secondly, the PINs contain a unique amphiphilic tryptophan rich domain (TRD) that is not found in the ns-LTPs or alpha-amylase inhibitors (Blochet et al., 1993; Gautier et al., 1994). The Trp residues occupy a surface loop and are proposed to comprise the membrane lipid-binding site (Le Bihan et al., 1996; Kooijman et al., 1997). PINA has a higher ability than PINB to form aggregates under certain conditions, and this involves the TRD, similar to the involvement of a TRD in oligomerisation of a membranotoxin secreted by a bacterium (Le Bihan et al., 1996). The PINs are able to bind to bilayer vesicles, with a preference for anionic phospholipid vesicles over neutral polar ones (Dubreil et al., 1997). Two types of interactions are proposed to occur between PINs and membrane lipids; hydrophobic interactions between Trps of TRD and the membrane lipid tails, and electrostatic interactions between the arginine and lysine residues flanking the Trp residues and the phosphate head-groups of membrane lipids (Kooijman et al., 1997). It appears that PINs are able to penetrate the membranes more deeply due to their unique TRD, compared to the weaker membrane interface-only binding of ns-LTPs (Le Guerneve et al., 1998; Douhiez et al., 2000) and the mechanism of cytotoxicity of PINs is proposed to involve membrane perturbation (Le Guerneve et al., 1998). PINA (and α1-purothionin; see below) can form cation-selective channels, as shown by studies using giant liposomes (Llanos et al., 2006).

3.2 Antimicrobial activities of PINs:

A number of studies have confirmed the in-vitro or in-vivo antimicrobial activities of puroindolines and synthetic peptides based on these. The research in this area has been reviewed recently (Bhave and Morris, 2008b). In summary, the 13- and 12-residue synthetic peptides mimicking the TRDs of PINA and PINB (Jing et al., 2003), and the PIN proteins purified from wheat flour (Dubreil et al., 1998) and/or from bacterial systems expressing cloned Pin genes (Capparelli et al., 2006; 2007), have been tested against pathogenic strains of bacteria and fungi, including some human pathogens, and show various degrees of activities (Table 2). We recently tested a variety of PIN-based peptides not only against
laboratory strains but also fungi, and showed that certain residues are important for the activity, including those in the TRD (Phillips et al., 2011).

### Table 2: Antimicrobial properties of puroindolines and PIN-based peptides

<table>
<thead>
<tr>
<th>PIN peptide/protein</th>
<th>Active against</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified native PINA, PINB proteins</td>
<td><em>Alternaria brassicola</em>, <em>Ascohyta pisi</em>, <em>Botrytis cinerea</em>, <em>Verticillium dahliae</em>, <em>Fusarium culmorum</em></td>
<td>Marion et al., 1994; Dubreil et al., 1998</td>
</tr>
<tr>
<td>Purified native PINA protein</td>
<td><em>Erwinia amylovora</em></td>
<td>Mourguès et al., 1998</td>
</tr>
<tr>
<td>PuroA and puroB peptides based on TRD of PINA and PINB</td>
<td><em>Escherichia coli</em>, <em>Staphylococcus aureus</em></td>
<td>Jing et al., 2003</td>
</tr>
<tr>
<td>Purified native PINA and PINB proteins</td>
<td><em>Staphylococcus aureus</em>, <em>Clavibacter michiganensis</em>, <em>Escherichia coli</em>, <em>Agrobacterium tumefaciens</em>, <em>Erwinia carotovora</em>, <em>Pseudomonas syringae</em></td>
<td>Capparelli et al., 2005</td>
</tr>
<tr>
<td>PINA and PINB proteins expressed in <em>E. Coli</em></td>
<td><em>Escherichia coli</em>, <em>Staphylococcus aureus</em></td>
<td>Capparelli et al., 2006; 2007</td>
</tr>
<tr>
<td>Transgenic expression of wheat PINA protein in rice</td>
<td><em>Magnaporthe grisea</em>, <em>Rhizoctonia solani</em></td>
<td>Krishnamurthy et al., 2001</td>
</tr>
<tr>
<td>Transgenic expression of wheat PINB protein in apple</td>
<td><em>Venturia inaequalis</em></td>
<td>Faize et al., 2004</td>
</tr>
<tr>
<td>Transgenic expression of common wheat PINA in durum wheat</td>
<td><em>Puccinia triticina</em></td>
<td>Luo et al., 2008</td>
</tr>
<tr>
<td>Transgenic expression of wheat PINs in corn</td>
<td><em>Cochliobolus heterostrophus</em></td>
<td>Zhang et al., 2011</td>
</tr>
<tr>
<td>Various wild-type and mutant PIN-based peptides</td>
<td><em>Escherichia coli</em>, <em>Staphylococcus aureus</em>, <em>Collectotrichium graminicola</em>, <em>Drechslera brizae</em>, <em>Fusarium oxysporum</em>, <em>Rhizoctonia cerealis</em>, <em>Rhizoctonia solani</em></td>
<td>Phillips et al., 2011</td>
</tr>
</tbody>
</table>

"Fungi; "*"bacteria

Wheat Pin genes transgenically expressed in different plants that lack endogenous Pin genes, followed by challenging of the plants with appropriate pathogens, have confirmed in-planta activities, irrespective of whether the plant is evolutionarily highly related to wheat, e.g., durum wheat (Luo et al., 2008), or is relatively removed (remote?), e.g., rice (Krishnamurthy et al., 2001) or corn (Zhang et al., 2011), or is as distant as apple, a dicot (Faize et al., 2004) (Table 2). Collectively, the results strongly suggest the potential of PINs as novel biocidal agents for plant protection, and ectopic applications of the purified proteins or peptides for control of human infections.

## 4. Thionins

### 4.1 Biochemical properties of thionins:

Thionins comprise a group of small (approximately 5kDa) Cys-rich peptides that occur in a vast number of dicotyledonous and monocotyledonous plant species (Stec, 2006). Thionins are included in the pathogenesis-related (PR) proteins as the PR-13 group (Epple et al., 1995), due to their toxicity against phytopathogens and also their ability to accumulate in plants to contribute to the innate immunity. They are generally 45-47 amino acids long and are classified into 5 subclasses (type I to type V). Type I thionins (purothionins) were first reported from wheat grain endosperm, are 45 amino acids long, basic and contain 8 Cys (Fernandez de Caleya et al., 1972) and three different purothionins from wheat, i.e., α₁, α₂, and β, have been reported (Fernandez de Caleya et al., 1976; Egorov et al., 2005). Type II thionins are slightly less basic than type I, 46-47 amino acids long with four disulfide bonds and were first isolated from barley leaves (α-hordothionin and β-hordothionin) (Rodriguez-Palenzuela et al., 1988) and the leaves and nuts of *Pyrularia pubera* (a parasite from Santalaceae) (Vernon, 1992). Type III thionins (45-46 amino acids) were
isolated from stems and leaves of mistletoe species (Loranthaceae) such as *Viscum album* (viscotoxins A1, A2, A3, B2, 1-PS, U-PS, C1) (Samuelsson and Pettersson, 1970; Romagnoli et al., 2003; Pal et al., 2008), *Phoradendron tomentosum* (phoratoxins A, B), *Dendrophthora clavata* (denclatoxin B) and *Phoradendron liga* (ligatoxin A) (Thunberg and Samuelsson, 1982 and within). The Type IV thionin, Crambin, isolated from seeds of *Crambe abyssinica*, is 46 amino acids long, has an overall neutral charge and possesses three disulphide bonds (Vanetten et al., 1965). Type V thionin, also isolated from wheat endosperm, is neutral and homologous to type I thionins except for some alterations in the mature protein (Cas tagnaro et al., 1992). Hellethionin D falls under a family called hellet hionins, isolated from the roots of *Helleborus purpurascens*; the hellethionins have certain pharmaceutical applications (Milbradt et al., 2003).

The γ-thionins are proposed to share a common ancestor with α/β thionins but should be classified into plant defensins (a different class of plant AMPs) due to structural variations from α/β thionins (Stec, 2006).

The three-dimensional structure of crambin, purothionins, α- and β-hordothionin and the viscotoxins A3, A1 and B2 have been deciphered (Debrecceni et al., 2003; Johnson et al., 2005; Stec et al., 2006; Pal et al., 2008). The three-dimensional structure of purothionins and crambin is represented by the Greek letter Gamma (Γ), and is comprised of two α-helices (which form a helix-turn-helix motif), a C-terminal coil and two short β strands (that form a β-sheet). The tertiary structure is held together by four disulfide bonds, salt bridges and intramolecular hydrogen bonds in purothionins (reviewed in Stec et al., 2006), whereas crambin and the viscotoxins (except for viscotoxin U-PS) have only three disulphide bridges (Romagnoli et al., 2003). The groove between the α-helices and β-sheets possesses the Tyr13 residue, the membrane interactions of which may be associated with cell leakage (Cas tagnaro et al., 1981). Cell leakage appears to be a common mechanism of cell lysis of thionins (Hughes et al., 2000; Richard et al., 2002). Tyr13 is replaced by a Phe in crambin, which may explain why crambin is non-toxic (Stec, 2006). Many hypotheses have been proposed regarding the mechanisms of cell lysis by various thionins, e.g., ion channels (Hughes et al., 2000; Llanos et al., 2006), lipid rafts (Giudici et al., 2004) and solubilisation of membrane phospholipids (Stec et al., 2004). Recent studies with model membranes (Majewski and Stec, 2010) showed solubilisation to be the cause of membrane leakage. Most interestingly, Oard (2011) has proposed the formation of water channels, similar to aquaporins, by α-hordothionin, wherein the highly conserved Cys and Tyr residues form the pore wall, leading water molecules to the centre of the phospholipid bilayer, thus causing local membrane disruption.

### 4.2 Antimicrobial activities of thionins:

The activity of thionins against bacteria, yeast, fungi (Fernandez de Caley a et al., 1972), cultured mammalian cells (Nakanishi et al., 1979; Carrasco et al., 1981) and insect larvae (Kramer et al., 1979) is well-established (Table 3), and the thionins are now classified as the PR-13 group of pathogenesis-related proteins (Epple et al., 1995), as mentioned above. The early observations included inhibition of growth of several human and plant pathogenic bacteria (Fernandez de Caley a et al., 1972) (Table 3), with β-purothionin being an efficacious antifungal peptide (Garcia-Olmedo et al., 1998). Tests on *Neurospora crassa* in vitro showed α-purothionin caused membrane permeabilization (Thevissen et al., 1999). Wheat purothionins exhibited antifungal activity by membrane permeabilization against *Rhizoctonia solani*, responsible for rice sheath blight and significant crop losses (Oard et al., 2004). Further, Giudici et al. (2004) demonstrated very strong antifungal activity of viscotoxins A3 and B that inhibited spore germination and mycelial growth of three fungal species (Table 3).
Table 3. Antimicrobial activities of thionins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Active against</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Wheat endosperm crude purothionin</td>
<td><em>Pseudomonas solanacearum</em>, <em>Xanthomonas phaseoli</em>, <em>Xanthomonas campestris</em>, <em>Erwinia amylovora</em>, <em>Corynebacterium flaccumfaciens</em>, <em>Corynebacterium michiganense</em>, <em>Corynebacterium poinsettiae</em>, <em>Corynebacterium sepedonicum</em>, <em>Corynebacterium fascians</em></td>
<td>Fernandez de Caley et al., 1972</td>
</tr>
<tr>
<td>Transgenic tobacco expressing α-thionin gene from barley</td>
<td><em>Pseudomonas syringae</em> pv. tabaci 153*, <em>Pseudomonas syringae</em> pv. syringae*</td>
<td>Carmona et al., 1993</td>
</tr>
<tr>
<td>Transgenic rice expressing oat thionins</td>
<td><em>Bukholderia plantarii</em>, <em>Bukholderia glumae</em></td>
<td>Iwai et al., 2002</td>
</tr>
<tr>
<td>Wheat endosperm α-purothionin</td>
<td><em>Neurospora crassa</em>, <em>Rhizoctonia solani</em></td>
<td>Thevissen et al., 1999; Oard et al., 2004</td>
</tr>
<tr>
<td>Viscotoxin A3 and B from leaves and stems of <em>Viscum album</em> L.</td>
<td><em>Fusarium solani</em>, <em>Sclerotinia sclerotiorum</em>, <em>Phytophthora infestans</em></td>
<td>Giudici et al., 2004</td>
</tr>
<tr>
<td>Transgenic tobacco expressing barley β-hordothionin</td>
<td><em>Helicoverpa armigera</em> (tobacco budworm) larvae, <em>Botrytis cinerea</em>, <em>Pseudomonas solanacearum</em></td>
<td>Charity et al., 2005</td>
</tr>
<tr>
<td>Transgenic Arabidopsis expressing β-purothionin</td>
<td><em>Pseudomonas syringae</em> pv tomato*, <em>Fusarium oxysporum</em></td>
<td>Oard and Enright, 2006</td>
</tr>
<tr>
<td>Transgenic oat expressing barley hordothionin Hh1</td>
<td><em>Fusarium graminearum</em></td>
<td>Carlson et al., 2006</td>
</tr>
<tr>
<td><em>Nicotiana attenuata</em> PR-13/Thionins</td>
<td><em>Pseudomonas syringae</em> pv. tomato*</td>
<td>Rayapuram et al., 2008</td>
</tr>
<tr>
<td>Pearl millet seed Thionin</td>
<td><em>Sclerospora graminicola</em></td>
<td>Chandrashekara et al., 2010</td>
</tr>
</tbody>
</table>

*Fungi; **bacteria

A number of experiments involving transgenic expression of thionin genes also confirm in-planta activity. The barley α-thionin expressed in tobacco led to transgenic plants exhibiting enhanced resistance to two strains of the phytopathogen *Pseudomonas syringae* (Carmona et al., 1993). Enhanced antibacterial and antifungal activity was also confirmed in transgenic tobacco and Arabidopsis (Mitsuhara et al., 2000; Oard and Enright, 2006). Transgenic tobacco expressing β-hordothionin showed resistance to *Botrytis cinerea* (grey mould) and *Pseudomonas solanacearum* (bacterial wilt) (Charity et al., 2005), and barley hordothionin purified from transgenic oat inhibited the growth of *F. graminearum* (Carlson et al., 2006). *Nicotiana attenuata*, a native tobacco, is susceptible to *Pseudomonas syringae* pv. *tomato* and the plant defence system triggers the production of salicylic acid and upregulates two pathogenesis-related genes. PR-1 and PR-13 (thionins); Rayapuram et al. (2008) noted that the plant with RNAi-silenced PR-13 (thionin) withered under *Sringae* pv. *tomato* infection, showing a causative role for it in antibacterial activity. Thionin genes were found to be upregulated in the seeds of pearl millet, only in lines resistant to *Sclerospora graminicola* that causes downy mildew, and the peptide purified from resistant seeds was confirmed to be a thionin (Chandrashekara et al., 2010). The fluorescently labelled thionin was also observed to accumulate in the vascular tissue and epidermal regions in planta (Chandrashekara et al., 2010), thus supporing the inclusion of thionins into PR proteins.

5. Perspective

The discoveries of numerous antimicrobial proteins and peptides form plants, some of which are discussed below, provide a number of research and development directions. Numerous trials show the potential of ns-LTPs, PINs and thionins for in-planta pathogen resistance in diverse species. Likewise, trials involving purified proteins or synthetic peptides based on PINs suggest a strong potential for ectopic applications for conditions such as skin infections. Due to these proteins being from natural and most often edible sources such as seeds, it is conceivable that they could also be used for novel applications in water and food safety.
References


