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STABILITY OF PUROINDOLINE PEPTIDES AND EFFECTS ON WHEAT RUST

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Abstract

Peptides modelled on the tryptophan rich domain (TRD) for puroindolines and the related Grain Softness Protein-1 have a broad range of antibacterial and antifungal activities. With the aims of further investigating the activities of these antimicrobial peptides we studied their activity against wheat rust diseases and environmental stability. PINA-based peptides were found to have high pH and thermal stability in addition to being stable over long periods at room temperature. These properties could make them excellent candidates as preservatives in food. PuroA, Pina-R39G and PuroB peptides adversely affected the morphology of the stripe rust spores (*Puccinia striiformis* f. sp. *tritici*), while PuroA and PuroB showed moderate inhibition of their germination. Additionally, GSP-5D reduced the germination of leaf rust spores (*P. triticina*). PuroA and PuroB spayed onto stripe rust infected plants effected a moderate reduction in the number of stripe rust uredinia on wheat seedlings, as did PuroB sprayed onto the seedlings and allowed to coat the leaves for 5 day prior to spore infection. The results suggest that the presence of the PIN-based peptides may lower frequency of initial infection foci.

Key words

Antimicrobial peptides; Puroindolines; Grain Softness Protein-1; wheat rust; *Puccinia striiformis; Puccinia triticina*

Introduction

Antimicrobial peptides (AMPs) are a form of innate defence against pathogen attack in many organisms. Resistance to conventional antibiotics by microorganisms is an ever-increasing problem, and AMPs offer a viable alternative due to their broad spectrum activity and low potential for development of resistance (Hancock and Sahl, 2006). AMPs are typically composed of 12-60 amino acid residues, amphipathic, cationic and often have a high proportion of hydrophobic residues (Shai and Oren, 2001). Cationic AMPs are often highly selective towards bacterial and fungal membranes due to their negatively charged phospho-lipid headgroups, compared to the zwitterionic headgroups of plant and animal membrane lipids. The antimicrobial activity of such peptides is suggested to involve ionic interactions between their basic residues and the negatively charged phospholipid headgroups, and also possibly the lipopolysaccharides and teichoic acid of microbial membranes, followed by membrane permeabilisation by various mechanisms (reviewed in Shai, 2002; Jenssen et al., 2006).

The *Puroindoline-a* (*Pina*) and *Puroindoline-b* (*Pinb*) gene transcripts and puroindoline (PIN) proteins have been shown to be expressed only in developing wheat seeds, leading to the hypothesis that the *in planta* roles of PINs might involve defending the seed and young plant against microbial pathogens (Blochet et al., 1993). The microbial membrane interaction required for this effect most likely occurs via the lipid-binding properties of PINs (Blochet et al., 1993). These properties may also be central to the association of PIN proteins with starch granule membranes and effects on wheat grain texture (Wanjugi et al., 2007; reviewed in Bhave and Morris 2008a, b; Kim et al., 2012b). PINs are reported to exhibit antimicrobial activity *in vitro* against several bacteria and fungi (Dubreil et al., 1998; Capparelli et al., 2005; Miao et al., 2012) and transgenic plant work has confirmed their causative association

with antibacterial and antifungal effects (Krishnamurthy et al., 2001; Faize et al., 2004; Luo et al., 2008; Kim et al., 2012a). The synthetic 13-residue peptide PuroA (FPVTWRWWKWWKG-NH_2), based on the signature motif called the 'tryptophan rich domain' (TRD) of PINA, was identified as the bactericidal domain (Jing et al., 2003). PuroA binds strongly to membranes with negatively charged phospholipid head-groups. Its low haemolytic activity towards mammalian cells (Jing et al., 2003) suggests high selectivity for bacterial membranes, a feature shared with wild-type PIN proteins (Capparelli et al., 2007). Single naturally occurring mutations at the TRD of PINB (Gly46Ser and Trp44Arg) which have been associated with hard grain texture, were found to have a decreased selectivity towards anionic phospholipids (Clifton et al., 2007), and would likely decrease the antimicrobial activity. It has also been proposed that the Arg residue(s) adjacent to the Trp residue(s) in the TRD may influence the biochemical properties of the Trp and enhance their membrane insertion (Jing et al., 2003). If this applies, the 5 Trps and the adjacent Arg in PINA could result in more potent disruption of microbial membranes compared to PINB which has only 3 Trps and a Lys instead of Arg (Gautier et al., 1994). However, no systematic analysis has been conducted testing the relevance of the Trp and/or Arg residues within the TRD to the antimicrobial properties.

The distantly related protein, grain softness protein-1 (GSP-1), encoded by the *Gsp-1* genes, also has a highly similar structure, except for a TRD comprised of only two Trps (Rahman et al., 1994). However, there is no strong evidence for this protein in controlling grain texture, and neither has it been assessed for antimicrobial activity. Orthologs of *Pin* genes also occur in other Triticeae members e.g., oat (avenoindolines) and rye (secaloindolines) (Gautier et al., 2000) and barley (hordoindolines) (Darlington et al., 2001). There is little research at present on the textural differences in rye or oats. However, the textural difference in barley is of

significant commercial interests in beer production. The grains of soft-textured malting quality cultivars are considered better suited for malting (Brennan et al., 1996; Psota et al., 2007). The hordoindolines (HINs) have been implicated in endosperm textural control (Beecher et al., 2002; Turuspekov et al., 2008; Galassi et al., 2012), and confirmed when an *hordolindoline b-2* null mutant was found associated with increased hardness (Takahashi et al., 2010).

We have recently shown that a number of peptides with sequences based on the TRD of PINs and HINs are active against bacteria and phytopathogenic fungi (Phillips et al., 2011). However, they have not been tested against the major cereal pathogen causing rust diseases (*Puccinia* species) which can lead to significant loss of yield. This work explores the effects of the Pin-based peptides on two main rust diseases of wheat to enhance our understanding of the membrane-binding ability of these proteins, an aspect which is intricately related to their food technological applications and possibly any in-vivo roles.

Materials and methods

Design and synthesis of peptides: Custom peptides PuroA and PuroB were modelled on the TRD of the PINs encoded by the wild-type alleles *Pina-D1a* and *Pinb-D1b,* respectively. Pinb-B and Pina-M were designed based on TRD of the natural hardness-associated alleles *Pinb-D1b* (Giroux and Morris, 1997) and *Pina-D1m* (Chen et al., 2006), respectively, while Pina-R39G was designed to investigate the role of Arg-39 adjacent to the Trp in any antimicrobial activity. Peptide GSP-5D was based on the wheat Grain Softness Protein-1 (encoded by Gsp-1 on chromosome 5D; Chantret et al., 2005, CR626934). Indolicidin, the unrelated well-studied Trp-rich antimicrobial peptide (AMP), was used as a 'positive control' due to its known activity (Selsted et al., 1992; Lee et al., 2003). The isoelectic points (pI) were predicted using the 'compute pI/MW Tool' at the Expert Protein Analysis System

(http://au.expasy.org/tools/pi_tool.html). The peptides ranged in length from 12 to 14 residues, and were cationic, with a net charge of +2 or +3 and pI of 10.00 to 12.01 (Table 1). All peptides were synthesised to >95% purity by Biomatik Corp (Ontario, CA) by solid-phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry and with C-terminal amidation (NH_2). Stock solutions were prepared at 1 mg/mL in 0.01% glacial acetic acid and stored at -20°C and dilutions to various concentrations were made in 0.01% acetic acid for antimicrobial testing. Alternatively, dilutions were made in 0.01% Tween-20 for better dispersion for the peptides to be sprayed onto the surface of leaves.

Antibacterial activity assays: The minimum inhibitory concentrations (MIC) of the peptides were determined against *Escherichia coli* (ATCC 25922) by the microtitre broth dilution method, as described previously (Phillips et al. 2011). In brief, the bacteria were grown in Mueller-Hinton Broth (MHB; Oxoid) at 37°C with shaking and the cell suspension adjusted to 5 x 10^5 CFU/mL in MHB. Peptides in 25 µL volumes were added to sterile, 96-well, flat-bottom polypropylene plates and a two-fold serial dilution conducted across each row, from 250 to 0.5 µg/mL. The wells were then inoculated with 75 µL of cell suspensions and incubated overnight at 37°C. All peptides were tested in triplicate. The MIC values were defined as the lowest peptide concentration that completely inhibited bacterial growth, and determined by measuring absorbance at 595nm using a Microplate reader (Bio-Rad, USA).

Peptide stability testing: The PuroA and Pina-M solutions (1 mg/mL) were incubated at 70°C, 80°C, 90°C, 100°C and 130°C, for 30 and 60 min at each temperature. After cooling on ice for 5 min, their activity against *E. coli* was measured as above. Separately, the peptide solutions were adjusted to pH ranging from 2 to 12 using 5M NaOH or 5M HCl, then

incubated at room temperature (RT) for 1 h and tested against *E. coli*. The peptide solutions (pH 7.0) were also left at RT for 8 weeks, followed by testing against *E. coli*.

Effect of peptides on wheat rust spore morphology: Mature plants of wheat (*T .aestiv*um) cv. Morocco, a susceptible variety, found naturally infected with stripe rust (*P. striiformis*) were obtained from the Department of Primary Industries (DPI) trials at Horsham, Victoria, Australia. A single leaf with heavy spore load was excised from an infected plant and approximately 0.5 x 1.0 cm sections were cut out from it. For assessing the general effect of peptides on spore morphology, PuroA, PuroB, GSP-5D, Pina-M, Pina-R39G or Pinb-B, peptide solutions (at a final concentration of 250 µg/mL) were made up in 200 µL volumes. Peptide solutions in 200 µL volumes were also made up for PuroA and PuroB at final concentrations of 125, 64 and 32 µg/mL to determine their approximate minimum inhibitory concentrations. Three leaf sections were added to each of these 200 µL solutions and incubated at RT for 48 h. At time zero (T_0), 24 h (T_{24}) and 48 h (T_{48}), slides were prepared for microscopy by removing a single leaf section from each peptide solution and gently rubbing the infected area onto a glass slide to transfer the spores. 10 µL of distilled water was added onto the spores, followed by placing a cover-slip. Spore morphology was observed by light microscopy at 400× magnification without staining. Spore-infected leaf sections treated with water or a commercial rust fungicide (Mycobutanil), were prepared as negative and positive controls, respectively, for antifungal activity tests.

Effect of peptides on wheat leaf rust spore germination: Noble agar (Difco-BD, USA) plates (51mm) were placed agar-side up in a spore inoculation chamber. Their surface was sprayed with rust spores (2.7 x 10^5 spores/mL) in light mineral oil and the spores allowed to settle for 10 min. 50 µL of PuroA, PuroB or GSP-5D peptide solutions at 250 to 32 µg/mL

concentrations were then spread on the agar surface, the plates wrapped in foil and incubated at 15°C with 80% humidity in an incubation chamber overnight. They were then examined by light microscopy at 100× magnification. For each plate, four fields were selected at random and the number of germinated and non-germinated spores recorded.

Treatment of wheat leaf rust with peptides: Seeds of wheat cv. Morocco were sown in potting mix and transferred to a plant growth cabinet, maintained at a constant humidity of 70% and temperature of 25°C, with simulated long day conditions (16 h/day of light). The 8 day old seedlings were then subjected to following treatments, each conducted in triplicate.

- **Treatment 1: Application of PuroA and PuroB peptides to seedlings, followed by leaf rust infection after 5 days**: A 250 µL aliquot of PuroA or PuroB solutions at 125 µg/mL in 0.01% Tween-20 was sprayed on the seedlings using a 1 mL mini-spray bottle, and the seedlings placed in a plant growth cabinet. Five days later they were inoculated with leaf rust (*P. triticina*) uredospores (suspended in light mineral oil at 2.7 x 10^5 spores/mL) and the spores allowed to settle for 2h. The uredospores had been obtained from leaves of a naturally infected wheat plant (PBI; Cobbitty, Australia). The plants were moved to a glass humidity chamber (80%) for 24h and then to a greenhouse for further growth at environmental conditions (typically a high of 20.5°C and low of 7.1°C for May, when the experiment was conducted).
- **Treatment 2: Application of PuroA peptide to seedlings at a higher concentration, followed by leaf rust infection after 5 days:** The seedling set-up was the same as above, but a higher concentration of PuroA (250 µg/mL) was applied.
- **Treatment 3: Infection of seedlings with leaf rust spores, followed by application of PuroA and PuroB peptides after 2 hours:** The seedlings were first inoculated with

uredospores and the spores allowed to settle for 2 h. The plants were then sprayed with 250 µL of PuroA or PuroB solutions at 125 µg/mL, placed in the humidity chamber (80%) for 24 h and then returned to the greenhouse.

- **Treatment 4: Infection of seedlings with leaf rust, followed by application of PuroA peptide after 5 days**: The seedlings were inoculated and the spores allowed to settle for 2h, as above. The plants were then moved to a humidity chamber (80%) for 24 h and then returned to the greenhouse. After 5 days, the seedlings were sprayed with 250 µL of PuroA or PuroB solutions at 125 µg/mL and then returned to the greenhouse.
- **No-peptide control**: The seedlings were inoculated and the spores allowed to settle, as above. The plants were placed in the humidity chamber (80%) for 24 h and then returned to the greenhouse.
- **Fungicide control:** The seedlings were inoculated and the spores allowed to settle, as above. The plants were sprayed with a commercial rust fungicide (Impact® active ingredient, Flutriafol 5 mg/mL), then moved to the humidity chamber (80%) for 24 h and then returned to the greenhouse.

All seedlings were assessed for the development of disease symptoms 13 days after inoculation, by scoring the infection type (IT) and disease severity on primary leaves. The ITs were recorded on the 0 to 4 scale of Roelfs and Martens (1988) as per the convention: 0 = immunity: no visible infection; 1 = small uredinia (pustules) often surrounded by necrosis; 2 = small or medium uredinia often surrounded by chlorosis; 3 = numerous uredinia of moderate size that may be associated with chlorosis or rarely necrosis; 4 = large uredinia without chlorosis or necrosis. The severity of infection was scored by enumerating the uredinia present on the primary leaves, as very low (VL) small uredinia; low (L): 1-5; moderate (M): 6-20; high (H): >20.

Results

The peptides used in this study were modelled on the TRD of puroindolines and the related Grain Softness Protein-1. All were cationic, with the predicted net charge of +2 or +3 and pI of 10.00 to 12.01 (Table 1). Selected peptides were then investigated for stability and/or activity against rust diseases.

The PIN peptides are stable at temperature and pH extremes: The MIC values of PuroA, PuroB and indolicidin against *E. coli* at 25^{o}C (Table S1) were found to be consistent with our previous work (Phillips et al., 2011) and other reports (Jing et al., 2003; Schibli et al., 2006). The results indicated a low bactericidal activity of PuroB (MIC >250 μg/mL) compared to that of PuroA (16 μg/mL) and Pina-M (13 μg/mL); hence the effects of pH and temperature were tested only on the latter two. Interestingly, both these peptides retained strong activity over a wide range of temperatures (70^{o}C - 130^{o}C) and pH (2.0 to 12.0), even after 1h incubations at these conditions (Table 2). Both also retained their activity after 1 week at RT. The activity dropped to 50% after 2 weeks, but then remained stable to the end of 8 weeks (the maximum time-frame investigated). The peptide IND only showed stable activity from pH 2.0 to 8.0, with a loss of activity at pH 10.0 and above.

The peptides affect the morphology of stripe rust spores: Rust fungi are biotrophic parasites of many plants including wheat, and hence cannot be cultured easily in liquid or solid media (Bolton et al., 2008). Hence the antifungal activity of the peptides was tested directly on spores infecting the wheat plants. The first approach investigated the effects on stripe rust (*P. striiformis*) spores from pustules present on excised sections of infected plants. The commercial foliar rust fungicide (Mycobutanil) was used as a positive control. Spores from samples treated with this exhibited no germination (lack of germ tubes) and a number of

morphological changes, the most notable being shrunken cytoplasm and the presence of fibrous material (probably representing denatured cellular material) compared to no-treatment control (Fig. 1A, B). Six peptides, PuroA, PuroB, GSP-5D, Pina-M, Pina-R39G and Pinb-B, were then tested at concentrations of 250 µg/mL. PuroA and PuroB were the most effective, and both induced morphological changes and inhibited the formation of germ tubes after 24 h incubation (Fig. 1C, D). Pina-R39G was partially active after 24 h, with only some spores exhibiting morphological changes (Fig. 1E), but it was more effective at 48 h, when all spores exhibited morphological changes and no germination (data not shown). Pina-M and Pinb-B showed no effects on the spore morphology or germination after 48 h. These peptides had Pro35Ser (Pina-M) and Gly46Ser (Pinb-B) substitutions, indicating that the presence of Ser may be detrimental to the activity against rust spores and also other phytopathogenic fungi (Phillips et al., 2011). GSP-5D also showed little effect, which may be attributable to its truncated TRD of only 2 Trp residues. The approximate 'minimum inhibitory concentration' of PuroA and PuroB against the stripe rust spores was then determined by testing their effects at 125, 64 and 32 µg/mL. PuroA was able to inhibit spore germination and induce morphological changes at 64 µg/mL, after 48 h treatment (data not shown). In contrast, PuroB exhibited limited activity at 125 µg/mL and negligible activity at 64 or 32 µg/mL. The higher sporicidal activity of PuroA compared to PuroB is consistent with the previously noted activities against filamentous fungi (Phillips et al., 2011).

Cultivation of wheat rust fungi in media (i.e., without the host plant) is not possible, but the viability of spores can be assessed by germination on solid media (Milus et al., 2006). Hence the spores of two major rust fungi, leaf rust (*Puccinia triticina*) and stripe rust (*P. striiformis*), were treated with PuroA, PuroB and GSP-5D peptide solutions of different concentrations on the surface of agar plates, followed by visualisation of germination using a light microscope

(data not shown). For the leaf rust spores, GSP-5D displayed the strongest activity with 56% inhibition of germination at 250 µg/mL and 28% inhibition at 64 µg/mL, compared with untreated sample (12% non-germination) (Table 3). PuroA showed significant inhibitory activity at 250 µg/mL (33%) but little activity at lower concentrations, and PuroB did not show any notable activity. The effect on germination of stripe rust spores was also investigated, at 250 µg/mL for all peptides (due to limited effects of PuroA at lower concentrations, as noted above). PuroA and PuroB showed the strongest activity (41% and 37% inhibition, respectively), compared to the no-treatment control, while GSP-5D showed limited effect (14.5% inhibition) (Table 3). This shows a degree of species-specificity or selectivity of peptides against certain pathogens.

In situ treatment of plants infected with rust diseases using peptides: The activity of PuroA and PuroB was also tested directly on leaf rust-infected wheat plants. The ITs were recorded using the 0 to 4 scale and the severity of infection was scored by enumerating the uredinia present on primary leaves, using the scale of VL, L, M and H, as described in Methods. Treatment 1, i.e., application of PuroA and PuroB peptides (at 125µg/mL) to uninfected wheat seedlings followed by inoculation with leaf rust spores 5 days later, showed that the seedlings pre-treated with PuroA had no decrease in symptoms, the primary leaves showing type 3 infection (moderate severity) at day 10, changing to type 4 infection (higher severity) at day 13 (Fig. 2A, B; Table 4). The seedlings pre-treated with PuroB showed a decrease in disease severity (a reduction in uredinia) at 10 and 14 days post inoculation, the primary leaves showing type 3 infection (but low severity) at day 10, changing to type 4 infection (at low to moderate severity) at day 13 (Fig. 2C, D; Table 4). In comparison, the no-peptide control showed moderate severity (type 3 infection) at day 10, progressing to high severity (type 4 infection) at day 13 (Table 4). Treatment 2 involved use of PuroA at a higher

concentration (at 250µg/mL), but did not lead to reduction in disease severity. Treatment 3 involved inoculation of seedlings with leaf rust spores first, followed by application of peptides (125µg/mL) 2 h later onto the relevant area, and showed that both peptides reduced the severity of infection at day 13 from high (in the no-peptide control) to low/moderate (Table 4). Treatment 4, which also involved inoculation of seedlings followed by application of PuroA 5 days later, showed no reduction in disease symptoms 13 days after infection.

Discussion

Tryptophan (Trp) residues are suggested to play a key role in the action of Trp-rich antimicrobial peptides such as tritrpticin and indolicidin, due to the unique amphipathic properties of its side chain (Schiffer et al., 1992). Our group (Phillips et al., 2011) and others (Jing et al., 2003; Ramalingam et al., 2012) have previously shown the antimicrobial properties of cationic peptides based on the unique TRD of the PIN proteins of wheat. These proteins are strictly localised to the wheat grain and have in fact been studied extensively for an unrelated reason; their major role in determining the commercially critical property of grain hardness (texture). This work explored the stability of the PIN-based cationic peptides under extreme conditions, as well as their activity against rust fungi, which are major pathogens of wheat, for further applications.

The results indicate that the PIN-based peptides are highly stable at pH and thermal extremes, as well as over long periods at room temperature, raising the possibility of their use as a preservative in various industries. For example, the heat stability could allow their use as a preservative of food products, and they may also enable lower processing temperatures, resulting in higher retention of food nutrients (Zhang et al., 2011). The stability at a wide range of pH likewise suggests potential for use in acidic or alkaline food products, and the

long term stability makes them excellent candidates as preservatives for foods stored at ambient temperatures (such as water or drinks), or for medical applications such as ectopic treatments of skin conditions, as reported (Capparelli et al., 2007). The high degree of stability is not unique amongst cationic AMPs; a bacteriocin purified from *Lactococcus lactis* was stable up over pH 1.0 to 11.0 and up to 100ºC, and retained >50% activity even after autoclaving (121ºC, 15 min) (Kumari et al., 2012). The fungal defensin spheniscin (40 amino acids) from the stomach of the King penguin (Landon et al., 2004) and plectasin (38 amino acids), produced by *Pseudoplectania nigrella* (Zhang et al., 2011) have similar thermal and pH stability, consistent with the stomach environment. The stability of defensins has been attributed to their high Cys content, the disulphide bonds providing structural support (Zhang et al., 2011). This finding may be relevant to the PINs which have a strong 10-Cys backbone, and suggests the feature of high stability may be shared with other ancestrally related proteins of the helicoid family (Le Bihan et al., 1996).

The antifungal activity testing of the PIN-based peptides (Phillips et al., 2011; Ramalingam et al., 2012) or PIN proteins (Dubreil et al., 1998) has focussed so far on in-vitro testing of cultures, *in planta* testing in plants transformed with *Pin* genes, e.g., rice (Krishnamurthy et al., 2001), apple (Faize et al., 2004) and durum wheat (Luo et al., 2008), and wheat seeds overexpressing *Pins* (Kim et al., 2012a). In an effort to test their effectiveness against one of the most common and severe diseases of wheat, the leaf rust (*P. triticina*) and stripe rust (*P. striiformis*), which can cause chlorosis or necrosis in the host plants (Bolton et al., 2008), we assessed their effect on the rust spores. The morphology and germ-tube formation of the stripe rust spores treated with PuroA, Pina-R39G and PuroB peptides were adversely affected, with the spores exhibiting shrunken or condensed cytoplasm and the appearance of 'fibres' (probably indicative of denatured cellular material). The germ-tubes, if present, were

truncated and many showed abnormal growth. The results support Barna et al. (2008) who showed the antifungal peptide PAF from *Penicillium chrysogenum* had a strong inhibitory effect on wheat leaf rust spores, including truncated and abnormal germ tubs (when present).

The germination of leaf rust and stripe rust spores treated with 250 µg/mL PuroA, PuroB and GSP-5D, as tested on agar plates, also showed clear inhibitory effects, the GSP-5D being most potent, reducing the germination to 44%. This was consistent with the strong activity of GSP-5D against phytopathogenic fungi noted earlier (Phillips et al., 2011). The results support our theory (Gollan et al., 2007) that *Gsp-1* may have a stronger role in pathogen protection, while the *Pin* genes have a more unique property of starch granule association that results in their effects on grain texture. PuroA had moderate inhibitory activity against the leaf and stripe rust spores, while PuroB had limited activity against stripe rust spores only. The results are consistent with our earlier results of lower antifungal activity of PuroB (MIC range 64-250 µg/mL) compared to PuroA (MIC range 32-250 µg/mL) (Phillips et al., 2011), possibly due to the fewer Trp residues in PuroB (Jing et al., 2003). In the leaf rust prevention experiments, the effect of peptides was assessed through a number of strategies. Spray application of the peptide solutions onto the leaves 2 h after infection with leaf rust spores was found to be the most effective treatment. When PuroA and PuroB were thus used, a moderate reduction in the number of uredinia present on the primary leaves was observed. A reduction in symptoms was also observed for PuroB when it was allowed to coat the leaves for 5 days prior to infection. The results suggest the presence peptides on the leaves may act directly on the spores and prevent the initial infection.

Although there is experimental data showing the *in vitro* antibacterial and antifungal activity of the PIN proteins (Dubreil et al., 1998) and PIN-based peptides (Phillips et al., 2011;

Ramalingam et al., 2012), the testing of *in vivo* activity has been limited to plants which lack *Pin* genes and have been genetically modified to express one or both *Pin* genes. Rice expressing wheat PINA and/or PINB exhibited a reduction in disease symptoms against rice blast (*Magnoporthe grisea*) and sheath blight (*Rhizoctonia solani*) (Krishnamurthy et al., 2001), apple plants expressing PINB showed an increased tolerance to scab (*Venturia inaequalis*) (Faize et al., 2004), PINA expressed in durum wheat enhanced its resistance to leaf rust (Luo et al., 2008) and over-expression of both PINs in wheat seeds reduced *Penicillium* sp. fungal infection (Kim et al., 2012a). In general, these reports showed that the PINs reduced or slowed the fungal growth but did not prevent the disease. In light of the varying effects of the peptides tested here on fungal spores and plants infected with spores, in-vivo expression of *Pin/Gsp-1* genes seems to have a strong basis for enhancing common and/or specific pathogen defences.

Many cationic AMPs are proposed to cause membrane permeabilisation by various methods (Shai, 2002; Jenssen et al., 2006), while others may translocate across membranes without permeabilising them, resulting in cell death by intracellular mechanisms (Subbalakshmi and Sitaram, 1998; Hsu et al., 2005). The PuroA and PuroB peptides tested here display high selectivity towards microbial cells and little toxicity to mammalian cells (Jing et al., 2003; Phillips et al., 2011). The PINA proteins are shown to induce membrane permeabilisation by the formation of membrane spanning ion channels in artificial and biological membranes (Charnet et al., 2003). However, it is unclear whether this mechanism also applies to all PIN-based peptides, including those with variant sequences, and their action on all bacterial and fungal membranes, or whether alternative permeabilisation mechanisms and/or intracellular actions may exist. Thus further explorations of the mechanisms of action of PIN peptides against microbial cells will be essential for their use as therapeutic or food safety agents.

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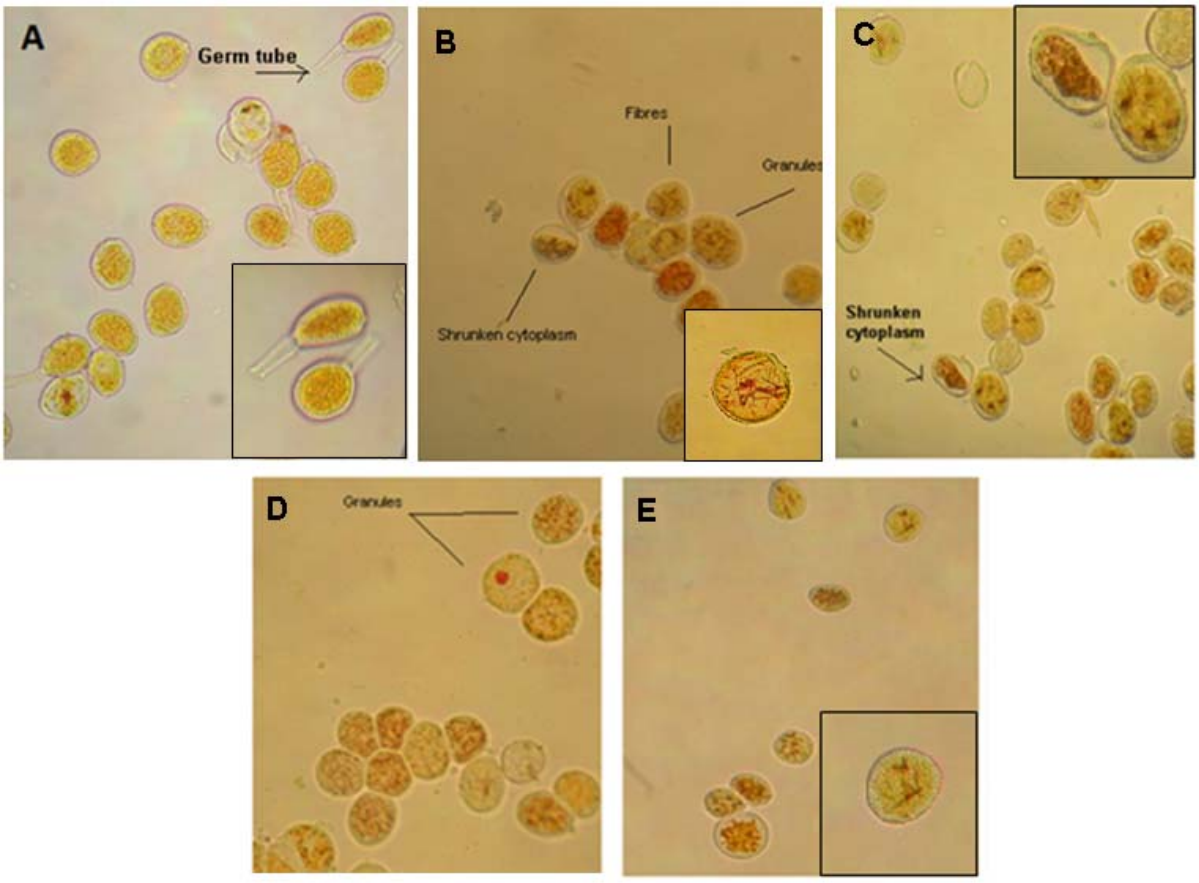

Figure 1. Effects of PIN-based peptides on the morphology of stripe rust spores.
A. Untreated spores from the surface of infected wheat leaves after 24 h incubation in water; **B.** spores treated with fungicide for 24 h; **C.** Spores treated with PuroA at 250µg/mL for 24 h; **D.** Spores treated with PuroB at 250µg/mL for 24 h; **E.** Spores treated with Pina-R39G at 250µg/mL for 24 h.

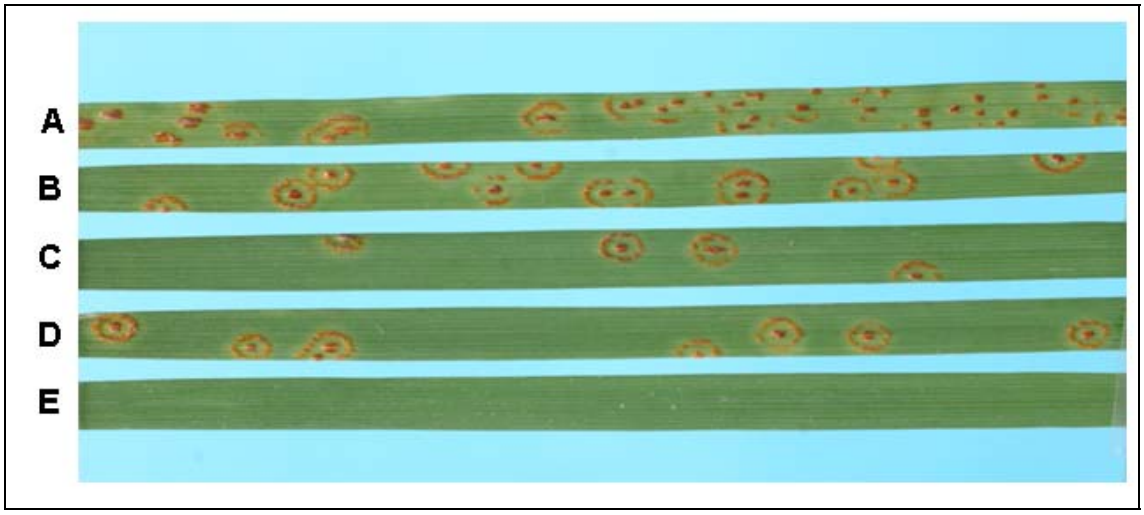

Figure 2. Leaf rust symptoms on primary leaf of wheat seedling 13 days after inoculation with spores.
A-B: Treatment 1; treated with 125µg/ml PuroA, infection type 4 with high severity of infection;
C-D: Treatment 1; treated with 125µg/ml PuroB, infection type 4 with low infection severity;
E: Treatment 1; treated with commercial fungicide, no infection.

Table 1. TRD-based puroindoline peptides tested for anti-rust effects

Peptide name	Sequence	MW[a]	Net charge	pI[b]	Change(s) to sequence	*Natural* allele with this TRD; GenBank/ Reference
PuroA	FPVTWRWWKWWKG-NH_2	1862.23	+3	11.17	Wild type	*Pina-D1a*; DQ363911
Pina-M	FSVTWRWWKWWKG-NH_2	1852.19	+3	11.17	Pro-35 → Ser[c]	*Pina-D1m;* EF620907
Pina-R39G	FPVTWGWWKWWKG-NH_2	1667.05	+2	10.00	Arg-39 → Gly[c]	N /A
PuroB	FPVTWPTKWWKG-NH_2	1531.84	+2	10.00	WT	*Pinb-D1a*; DQ363913
Pinb-B	FPVTWPTKWWKS-NH_2	1561.86	+2	10.00	Gly-46 → Ser[d]	*Pinb-D1b*; DQ363914
Pinb-D	FPVTWPTKWRKG-NH_2	1501.81	+3	11.17	Trp-44 → Arg[d]	*Pinb-D1d* Lillemo and Morris (2000)
GSP-5D	MPLSWFFPRTWGKR-NH_2	1808.20	+3	12.01	WT	*Gsp-1-5D;* CR626934
IND	ILPWKWPWWPWRR-NH2	1809.22	+3	12.01	WT	Selsted et al. (2002)

[a]Calculated using mass spectrometry data provided by Biomatik Corp (Ontario, Ca); [b]Predicted using the Compute pI/MW Tool' at ExPAsy (http://au.expasy.org/tools /pi_tool.html); [c]denotes the position of the substitution in relation to the mature puroindoline-a protein; [d] denotes the position of the substitution in relation to the mature puroindoline-b protein; IND: indolicidin.

Table 2. Effect of temperature and pH on the activity of peptides

	MIC (µg/mL)		
	PuroA[a]	**Pina-M[b]**	**IND[c]**
Temperature			
25°C	16	8	32
70°C	16	8	32
80°C	16	8	32
90°C	16	8	32
100°C	16	8	32
130°C	16	8	32
pH			
2.0	16	8	32
4.0	16	8	32
6.0	16	8	32
8.0	16	8	32
10.0	16	8	64
12.0	16	8	64

[a]MIC of PuroA: 16µg/mL, [b]MIC of Pina-M: 13±5µg/mL and [c]MIC of IND: 32µg/mL

Table 3. Effect of PIN-based peptides on wheat rust spore germination

	% inhibition of germination of leaf rust spores*				% inhibition of germination of stripe rust spores*
Peptide	250µg/mL	125µg/mL	64µg/mL	32µg/mL	250µg/ml
PuroA	33 (±7)	17 (±6)	13 (±3)	14 (±1)	41.1 (±9)
PuroB	18 (±4)	18 (±5)	11 (±1)	8.5 (±3)	37.5 (±8)
GSP-5D	56 (±3)	24 (±2)	28 (±5)	12.5 (±2)	14.5 (±2)
No-peptide control	12 (±3)	12 (±3)	12 (±3)	12 (±3)	12 (±2)

*determined as the mean of 3 experiments.

Table 4. *In situ* treatment of wheat leaf rust disease with PIN-based peptides

		Day 8 after seedling inoculation		Day 10 after seedling inoculation		Day 13 after seedling inoculation	
Peptide[a]	Plant[b]	Infection[c]	Severity[d]	Infection[c]	Severity[d]	Infection[c]	Severity[d]
Treatment 1: Application of PuroA and PuroB to seedlings, followed by leaf rust infection							
PuroA R1	P1	1	VL	3	M	4	H (>20)
	P2	1	VL	3	M	4	H (>20)
	P3	1	VL	3	M	4	H (>20)
PuroA R2	P1	1	VL	3	M	4	H (>20)
	P2	1	VL	3	M	4	H (>20)
	P3	1	VL	3	M	4	H (>20)
PuroB R1	P1	0	0	3	L	4	L (3)
	P2	0	0	3	L	4	L (5)
	P3	0	0	3	L	4	L (5)
PuroB R2	P1	0	0	3	L	4	M (15)
	P2	0	0	3	L	4	M (20)
	P3	0	0	3	L	4	L (3)
Treatment 2: Application of PuroA to seedlings at a higher concentration, followed by leaf rust infection							
PuroA R1	P1	0	0	3	M	4	H (>20)
	P2	0	0	3	M	4	H (>20)
	P3	0	0	3	M	4	H (>20)
PuroA R2	P1	0	0	3	M	4	H (>20)
	P2	0	0	3	M	4	H (>20)
	P3	0	0	3	M	4	H (>20)
Treatment 3: Infection of seedlings with leaf rust, followed by application of PuroA and PuroB after 2 h							
PuroA R1	P1	1	VL	3	L	4	L (2)
	P2	1	VL	3	L	4	L (3)
	P3	1	VL	3	L	4	L (4)
PuroA R2	P1	1	VL	3	M	4	M (6)
	P2	1	VL	3	M	4	M (6)
	P3	1	VL	3	M	4	H (>20)
PuroB R1	P1	1	VL	3	M	4	M (6)
	P2	1	VL	3	M	4	M (7)
	P3	1	VL	3	M	4	M (15)
PuroB R2	P1	1	VL	3	M	4	M (8)
	P2	1	VL	3	M	4	M (6)
	P3	1	VL	3	M	4	M (9)
Treatment 4: Infection of seedlings with leaf rust, followed by application of PuroA peptide after 5 days							
PuroA R1	P1	0	0	3	M	4	H (>20)
	P2	0	0	3	M	4	H (>20)
	P3	0	0	3	M	4	H (>20)
PuroA R2	P1	1	VL	3	M	4	H (>20)
	P2	1	VL	3	M	4	H (>20)
	P3	1	VL	3	M	4	H (>20)
No-peptide control							
	P1	0	0	3	M	4	H (>20)
	P2	0	0	3	M	4	H (>20)
	P3	0	0	3	M	4	H (>20)
Commercial fungicide control							
	P1	0	0	0	0	0	0
	P2	0	0	0	0	0	0
	P3	0	0	0	0	0	0

[a]All treatments were conducted with 2 independent technical replicate experiments (R1, R2); [b]each replicate experiment consisted of biological triplicates (plants P1, P2, P3); [c]type recorded using the 0 to 4 scale of Roelfs and Martens (1988) (see methods for explanation of scale); [d]scored by counting the uredinia on primary leaves (see methods for explanation of scale).