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Expression and functional characterization of the plant antimicrobial snakin-1 and defensin recombinant proteins

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ABSTRACT

In this study, for the first time, functionally active, recombinant, cysteine-rich plant proteins snakin-1 (SN1) and defensin (PTH1) were expressed and purified using a prokaryotic expression system. The overall level of antimicrobial activities of SN1 and PTH1 produced in *Escherichia coli* was commensurate with that of the same proteins previously obtained from plant tissues. Both proteins exhibited strong antibacterial activity against the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (50% inhibitory concentration (IC_{50}) 1.5–8 μM) and antifungal activity against the phytopathogenic fungi *Colletotrichum coccoides* and *Botrytis cinerea* (IC_{50} 5–14 μM). Significantly weaker activity was observed against *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *tabaci*. A pronounced synergistic antimicrobial effect against *P. syringae* pv. *syringae* and an additive effect against *P. syringae* pv. *tabaci* occurred with a combination of SN1 and PTH1. Aggregation of *C. michiganensis* subsp. *sepedonicus* bacterial cells at all protein concentrations tested was observed with the combination of SN1 and PTH1 and with SN1 alone. Our results demonstrate the use of a cost effective prokaryotic expression system for generation and *in vitro* characterization of plant cysteine-rich proteins with potential antimicrobial activities against a wide range of phytopathogenic microorganisms in order to select the most effective agents for future *in vivo* studies.

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Phytopathogenic bacteria and fungi cause significant losses in important agricultural crops and are the primary cause of postharvest diseases of fruits and vegetables. Postharvest losses are estimated to range from 10% to 30% per year despite the use of modern storage facilities and new crop conservation techniques [1]. There are several ways to overcome this problem, including application of chemicals [2], biological control [3], the induction of natural plant defenses [4] and crop biotechnology [5]. However, environmental safety problems, emergence of new virulent strains of pathogens, resistance to the current chemical and biological defenses, as well as practical difficulties, prevent full realization of the aforementioned strategies under commercial conditions and warrant development of new and more effective plant protection techniques.

Recently the use of antimicrobial peptides, naturally produced by a variety of microorganisms and plants, was proposed for bioengineering and crop protection applications [6–9]. The decrease of susceptibility to pathogen infection as a result of transgenic production of antimicrobial agents in plants has been reported for thionins [10,11], lipid transfer proteins (LTPs) [12], defensins [13,14], snakins [15,16], and *Leonurus japonicus* antimicrobial protein (LJAMP1) [17], which have been isolated from such plants as: *Vigna unguiculata*, *Arabidopsis thaliana*, *Medicago sativa*, *Raphanus sativus*, *Solanum tuberosum* and *Leonurus japonicus*. The natural production of such antimicrobial proteins in plants is thought to be part of the constitutive or inducible defense mechanisms against pathogens acquired by plants in the course of evolution [18]. To characterize the antimicrobial properties of the proteins listed above, a wide range of bacterial and fungal phytopathogens, such as *Erwinia chrysanthemi*, *Clavibacter michiganensis*, *Pseudomonas cichorii*, *Ralstonia solanacearum*, *Alternaria alternata*, *Alternaria brassicae*, *Aspergillus flavus*, *Aspergillus niger*, *Bipolaris maydis*, *Botrytis cinerea*, *Botrytis maydis*, *Cercospora personata*, *Colletotrichum gloeosporiodes*, *Colletotrichum lagenarium*, *Fusarium solani*, *Fusarium oxysporum*, *Magnaporthe grisea*, *Pyricularia grisea*, *Rhizoctonia cerealis*, *Rhizoctonia solani*, *Trichoderma harzianum* and *Verticillium dahliae*, have been tested. *Solanum lycopersicum*, *Solanum tuberosum*, *Nicotiana benthamiana* and *Nicotiana tabacum* have been used for genetic transformation, expression, and purification of recombinant antimicrobial proteins for subsequent microbiological experiments *in vitro*, and for studies on the antimicrobial properties of these proteins *in vivo* [11,13,15]. It is important that the selected proteins be tested on a wide variety of phytopathogenic

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microorganisms prior to performing plant transformation experiments, which can be expensive, time-consuming and potentially hindered by the limitations in the production of large quantities of antimicrobial proteins, which typically require large amount of plant tissue as a source of the biological material.

Prokaryotic protein expression is the most frequently used method to produce recombinant proteins because it meets all the above mentioned criteria, namely, is inexpensive, facilitates protein expression by its relative simplicity and allows achievement of high density cultivation in a relatively short period of time. Furthermore, there is currently a plethora of commercially available kits designed for recombinant protein expression in bacteria. However, expression of recombinant proteins in *Escherichia coli* often results in accumulation of the target proteins in the form of insoluble aggregates known as inclusion bodies (IBs). IBs usually consist of almost pure, aggregated proteins which are typically misfolded and thus biologically inactive [19]. In spite of the numerous known reasons for the IB formation, to date there has been no universal technique for efficient folding of the aggregation-prone recombinant proteins [20,21]. Although there are few examples where formation of IBs is highly desirable, such as their use for oral vaccination as an alternate route for the delivery of recombinant antigens for immunization [22], in most cases obtaining a recombinant protein in the soluble form is absolutely crucial.

A number of techniques allowing for re-distribution of proteins from IBs into the soluble cytoplasmic fraction have been described in the literature. Those can be divided into the procedures involving direct refolding of proteins from IBs [23], using modified washing, solubilization, and folding conditions, and those based on modified protein expression strategies to generate a recombinant protein in its soluble form [24]. The latter involve protein expression under reduced temperatures (utilization of the *csp*A promoter) [25], co-expression of the target proteins with chaperones (Cpn60 and Cpn10) which allows *E. coli* to grow at 4 °C [26], the use of special strains of *E. coli*, such as C41(DE3) and C43(DE3), to improving expression of the soluble form of recombinant proteins [27], modification of superproducing strain cultivation conditions [28], and the use of protein fusion technology to co-express molecular chaperones [29–31]. In spite of these modifications, the majority of these approaches do not fully guarantee increased solubility of recombinant proteins. Therefore, individual approaches need to be applied to solve the problems related to IB formation and solublization of each recombinant protein.

In the present study, we demonstrate the feasibility of generating functionally active antimicrobial plant proteins snakin-1 (SN1)[1] [16] and defensin (PTH1) [32] in the pET-vector based expression system. Attempts to express similar proteins in *E. coli* have failed [14]. The aim of this study was to assess the antimicrobial potential of SN1 and PTH1 obtained from a prokaryotic expression system.

Materials and methods

Cloning of potato sn1 and pth1 genes

The coding regions of the potato snakin-1 (*sn1*) [16] and defensin-1 (*pth1*) [32] genes were amplified from plasmid pP2C2S/SAP (kindly provided by Dr. Y. Zhao) using PCR and primer pairs corresponding to the N- and C-termini of the SN1 (SN1F/SN1R) and PTH1 (PTH1F/PTH1R) proteins. The following N-terminal primers were used—SN1F: 5′-dCTTAGCCATGGCTGGTTCAAATTTTTGTG-3′ and PTH1F: 5′-dCTTAGCCATGGTTAGACATTGCGAGTCG-3′ (Nco1 site is underlined), respectively. The following C-terminal primers were used—SN1R: 5′-TAAGAAGCTTTTATCAAGGGCATTTAGACTTGCCCTT-3′ and PTH1R: 5′-TAAGAAGCTTTTATCAGCATGGCTTAGTGCAAAAGCA-3′ (HindIII site is underlined), respectively. Carboxy-terminal 6×His-tagged proteins containing a thrombin cleavage recognition sequence (LeuValProArgGlySer) were designed to facilitate purification using a Ni–NTA resin with subsequent removal of the 6×His-tag from the expressed target proteins by site-specific thrombin. The following C-terminal primers were used to produce these proteins: (SN1thrHisR:5′-TAAGAAGCTTTTATCAGTGATGGTGATGGTGATGGCTGCCGCGCGGCACCAGAGGGCATTTAGACTTGCCCTT-3′ and PTH1thrHisR: 5′-TAAGAAGCTTTTATCA*GTGATGGTGATGGTGATG*GCTGCCGCGCGGCACCAGGCATGGCTTAGTGCAAAAGCA-3′ (HindIII site is underlined, thrombin recognition sequence—*italics*). The PCR products were cloned into the pCR®II-TOPO® Vector (Invitrogen, Carlsbad, CA), resulting in recombinant plasmids pCRsn1, pCRpth1, pCRsn1thrHis, and pCRpth1thrHis. These plasmids were subsequently digested with NcoI and HindIII and the corresponding restriction fragments were cloned into the plasmid vector pET26b+ (Novagen, Madison, WI) at the NcoI/HindIII sites, giving rise to pET26b+sn1, pET26b+pth1, pET26b+sn1thrHis, and pET26b+pth1thrHis, respectively. All constructs were verified by direct DNA sequencing.

Protein expression and characterization

Escherichia coli strain BL21 (DE3) (Stratagene, La Jolla, CA) was used as a host for expression of the target genes. The transformation of pET26b+sn1, pET26b+pth1, pET26b+sn1thrHis, and pET26b+pth1thrHis into *E. coli* was performed according to the manufacturer's instructions. The induction procedure for gene expression was as follows: 2 ml of Luria–Bertani broth (LB) [20 g of Bacto tryptone; 10 g of Bacto yeast extract and 20 g of NaCl per liter of H_2O] containing kanamycin (50 μg/ml) was inoculated with a bacterial colony and incubated overnight at 225 rpm at 37 °C. Five hundred microliters of overnight culture were transferred into a flask containing 50 ml of LB medium with the same antibiotic (50 μg/ml), and agitated at 225 rpm at 37 °C until the culture density reached an OD_{600} of 0.7–0.8. IPTG was added to final concentration 1.5 mM with subsequent incubation at 225 rpm at 37 °C for 7 h. After incubation, the bacterial cells were harvested by centrifugation in 50 ml Falcon tubes at 4000 rpm for 20 min at 4 °C and frozen at −80 °C. A non-induced culture was used as a negative control.

Protein extraction

The BugBuster Master Mix Protein Extraction Reagent (Novagen) was used to extract the expressed proteins from bacterial lysates. The extraction was carried out according to the manufacturer's instructions.

IB purification, solubilization and refolding

IBs were purified using BugBuster Master Mix Protein Extraction Reagent (Novagen) according to a standard procedure (Novagen, User protocol TB245 Rev. E 0304, P.8). Solubilization of the subsequent IBs was carried out using the Protein Refolding Kit (Novagen). Specifically, the IBs solubilization buffer was supplemented with 1 mM DTT for correct folding of disulfide bonds and with 2% *N*-lauroylsarcosine to reduce hydrophobic aggregation. The solubilized proteins were dialyzed against 20 mM Tris–HCl pH 8.0 four times at 4 °C. The first and second dialysis buffers were supplemented with 0.1 mM DTT.

Aliquots of the SN1 and PTH1 proteins were subjected to denaturing electrophoresis in a gradient Novex® Tris–Glycine Gel (10–20%; Invitrogen, Carlsbad, CA) according to the manufacturer's

[1] *Abbreviations used:* SN1, snakin-1; LTPs, lipid transfer proteins; LJAMP1, *leonurus japonicus* antimicrobial protein; IBs, inclusion bodies; LB, Luria–Bertani broth; PDA, potato dextrose agar.

instructions and visualized by staining with SimplyBlue Safe Stain (Invitrogen).

The protein concentration was measured with the Quick Start™ Bradford Dye Reagent (Bio-Rad, Hercules, CA) using a standard method [33] and a microplate reader 680 (Bio-Rad). All the extracted proteins were stored at −20 °C in 50% glycerol.

Protein purification with nickel–nitrilotriacetic acid (Ni–NTA) metal-affinity chromatography matrices

The Ni–NTA Spin Kit (QIAGEN, Valencia, CA) was used for purification of the target proteins. The purification was carried out according to the manufacturer's instructions.

Western blot analysis

Twelve microliters of the soluble SN1thrHis (1.9 mg/ml) and PTH1thrHis (2.3 mg/ml) were loaded on a gradient Novex® Tris–Glycine Gel (10–20%) and transferred to a nitrocellulose membrane (Invitrogen). The membrane was probed with a 1:3000 dilution of monoclonal anti-polyhistidine clone HIS-1 (mouse IgG2a isotype) antibodies (Sigma, Saint Louis, MO) followed by a 1:5000 dilution of goat anti-mouse phosphatase-labeled Antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The membrane was developed by utilizing the BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Inc.).

Microorganisms

The following phytopathogenic microorganisms were used: *Clavibacter michiganensis* subsp. *sepedonicus* AS1 (I.-M. Lee, Molecular Plant Pathology Laboratory, United States Department of Agriculture, Beltsville, MD, US); *Pseudomonas syringae* pv. *syringae* 61, *Pseudomonas syringae* pv. *tabaci* 11528 Race 0 (C.J. Baker, Molecular Plant Pathology Laboratory, United States Department of Agriculture, Beltsville, MD, US); *Collectotrichum coccoides* and *B. cinerea* (R. Jones, Genetic Improvement of Fruit and Vegetables Laboratory, United States Department of Agriculture, Beltsville, MD, US).

Clavibacter michiganensis subs. *sepedonicus*, a gram-positive bacterium, was grown on nutrient-broth yeast extract agar (NBY) [8.0 g of nutrient-broth (Difco, Detroit), 2.0 g of yeast extract, 2.0 g of K_2HPO_4, 0.5 g of KH_2PO_4, 2.5 g of glucose and 15.0 g of Bacto agar per 1 L of H_2O; after auto claving, 1.0 ml of a sterile solution of 1 M $MgSO_4$ was added]. After inoculation of solid medium with the bacterium, the plates were incubated for five days at 28 °C. The colonies from the plate were transferred into a culture tube containing NBY broth and grown at 28 °C with vigorous shaking (250 rpm) for 192 h.

Pseudomonas syringae pv. *syringae* and *P. syringae* pv. *tabaci*, gram-negative bacteria, were grown in KB medium previously described by King et al. [34] (KB) [20.0 g of Proteose peptone #3 (Difco, Detroit, MI), 1.5 g K_2HPO_4, 15.0 ml glycerol, 15.0 g Bacto agar per liter H_2O; after autoclaving, 6.0 ml of a sterile solution of 1 M $MgSO_4$ was added]. The culture plates were incubated for two days at 28 °C. The colonies from the plate were transferred into a tube containing KB broth and grown at 28 °C with shaking (250 rpm) overnight.

C. coccoides and *B. cinerea* were routinely cultured on potato dextrose agar (PDA) (Difco) plates for approximately 14 days at room temperature. For antifungal assays, the spores were collected and suspended in PDB (Sigma–Aldrich, Inc., St. Louis, MO). Spore concentrations were determined using a hemacytometer and a light microscope.

Antibacterial assays

The antibacterial activities of the purified proteins were determined by counting bacterial CFU (colony forming units). Inhibition assays were performed in sterile 10 ml Falcon tubes (BD, Franklin Lakes, NJ). Each protein concentration was tested with three replications. Bacterial suspensions were combined with a fixed volume of the proteins, adjusted by the appropriate medium to a particular concentration. The bacterial concentration at the beginning of experiment was 1×10^4 CFU per ml for *C. michiganensis* subsp. *sepedonicus*, 4×10^5 CFU per ml for *P. syringae* pv. *syringae* and 8×10^4 CFU per ml for *P. syringae* pv. *tabaci*. The final protein concentrations used in all antibacterial assays were 0, 1, 2, 7 and 14 μM. The total volume of the protein–bacterial mix was 1 ml. Tubes were incubated at 28 °C with continuous shaking at 250 rpm for 192 h and 48 h for *C. michiganensis* subsp. *sepedonicus* and pseudomonads, respectively. Following incubation, 100 μl aliquots of protein-treated bacterial cultures were serially diluted in sterile water (from 10^{-2}- to 10^{-6}-fold) and 25 μl of diluted bacterial suspensions were spread onto the appropriate solid medium. The plates were incubated at 28 °C and examined for bacterial growth by counting CFU at 5 and 2 days for *C. michiganensis* subsp. *sepedonicus* and pseudomonads, respectively. This scheme was applied for both SN1 and PTH1 proteins.

Antifungal assays

The antifungal activities of the purified proteins were determined by counting germinating and non-germinating protein-treated fungal spores. The fungal spores were prepared in PDB. For the inhibition assays, spore suspensions of 1×10^5 spores/ml of *C. coccoides* and *B. cinerea* were mixed in 1.5 ml test tubes with SN1 and PTH1 proteins adjusted by PDB so that final concentrations were 0, 1, 2, 7 and 14 μM. Each antifungal assay was performed in three replicates. The total volume of protein–fungus combination was 50 μl. Twenty-five microliters of the mix of each variant was applied on the surface of a hemacytometer, which was placed into a humid chamber (Petri dish with wet filter paper). The inhibitory activity on fungal spore germination was determined after 12 h of incubation at 28 °C for *C. coccoides* and 14 h of incubation at room temperature for *B. cinerea* by visualization using a light microscope. This scheme was applied for both proteins.

Results

Expression of SN1 and PTH1 proteins in E. coli

To obtain purified SN1 and PTH1 recombinant proteins and to determine their antimicrobial activity, we constructed expression cassettes pET26b+sn1 and pET26b+pth1, which were transformed into *E. coli* BL21 (DE3) cells for protein expression. The expression vector pET26b+ was chosen because it provides the N-terminal *pelB* signal sequence to the expressed proteins, which should facilitate their localization to the periplasm and reduce or eliminate IB formation. However, analysis of the total and soluble fractions of the expressed SN1 and PTH1 proteins by gel electrophoresis demonstrated that SN1 and PTH1 were localized exclusively in the insoluble fraction (IBs) (Fig. 1).

Our attempts to express the proteins at a lower temperature (25 °C) in order to avoid IB formation or extraction of the proteins from IBs using sonication or solubilization by 8 M urea failed as well. The IBs containing SN1 and PTH1 were purified and solubilized as described (see Materials and methods). When the solubilized IBs were analyzed by gel electrophoresis, the sizes of the proteins were consistent with the predicted molecular masses of SN1 and PTH1 (Fig. 2).

We also engineered SN1 and PTH1 with a carboxy terminal, 6× histidine fusion containing an embedded thrombin cleavage site in order to facilitate purification, creating SN1thrHis and PTH1thrHis,

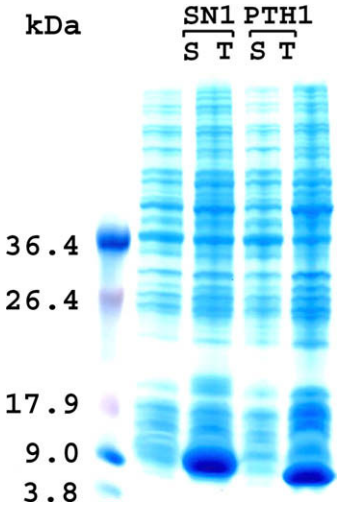

Fig. 1. Denaturing 10–20% polyacrylamide gel electrophoretic analysis of soluble (S) and total (T) fractions of SN1 and PTH1 expressed in *E. coli*. Sample lanes contain either 2 μg (S) or 4 μg (T) protein for both SN1 and PTH1. kDa = Precision Plus Protein Kaleidoscope standards (Bio-Rad). The gel was stained with SimplyBlue Safe Stain (Invitrogen).

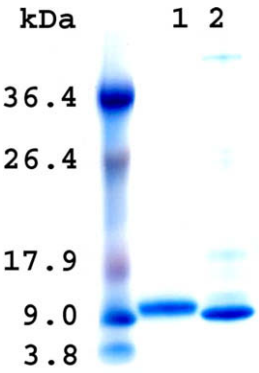

Fig. 2. Electrophoretic analysis of SN1 and PTH1 proteins after IB purification, solubilization, and protein refolding, as in Fig. 1. kDa = Precision Plus Protein Kaleidoscope standards (Bio-Rad). Lane 1 – SN1, 5 μg; Lane 2 – PTH1, 8 μg. The gel was stained with SimplyBlue Safe Stain (Invitrogen).

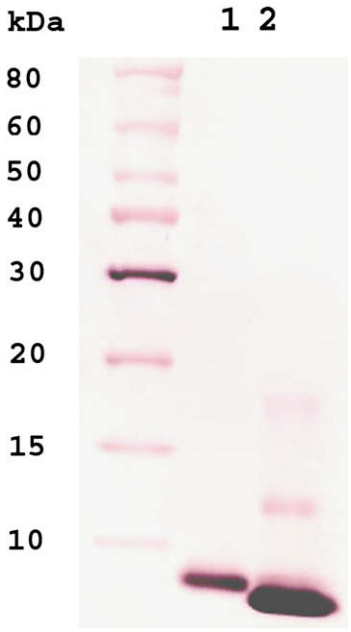

Fig. 3. Western blot analysis of SN1thrHis and PTH1thrHis after IB solubilization and protein refolding. The membrane was incubated with a monoclonal anti-polyhistidine clone HIS-1 (mouse IgG2a isotype) antibody followed by goat anti-mouse IgG as described in Material and Methods. kDa = BenchMark™ His-tagged protein standards (Invitrogen). Lane 1 – SN1thrHis, 23 μg; Lane 2 – PTH1thrHis, 28 μg.

respectively. Attempts to purify the resulting proteins after IB solubilization and protein refolding failed as well. To verify the presence of the polyhistidine tags in SN1 and PTH1 these proteins were subjected to Western blot analysis using a monoclonal anti-polyhistidine antibody. The results confirmed the presence of polyhistidine on both expressed proteins (Fig. 3). Thus, we believe that our inability to affinity-purify the target proteins using the Ni–NTA spin column could be accounted for by some conformational alterations in those proteins following their refolding, subsequently hiding the polyhistidine tags inside the protein structure and rendering them inaccessible for binding to the resin. Based on this observation the SN1 and PTH1 protein variants without polyhistidine were used for all the microbiological assays.

Attempts to analyze the structural properties of our proteins by analytical reverse phased HPLC and mass spectrometry failed (data not shown). For example, there were extremely heterogeneous peaks in one sample, with the most abundant peak appearing at 25 kDa. As our samples were prepared for optimum activity in the antimicrobial assays (purification from inclusion bodies and refolding) and not for physical evaluation, it may be that the proteins remained aggregated. Alternatively, the cysteine-rich nature of the proteins may interfere with the analyses. In addition, the protein concentrations of the solubilized peptides were determined using the Bradford reagent, and visual inspection of the gradient Novex® Tris–Glycine Gel (10–20%) (Fig. 2) revealed that the majority of the proteins in the sample lanes are the recombinant peptides. Therefore, protein concentrations calculated using the Bradford reagents were used to determine the molarity of peptides for the antimicrobial assays, but may not reflect the actual amount of functional protein in the samples.

Analysis of the antimicrobial activity of SN1 and PTH1

To assess the antimicrobial activities of the purified SN1 and PTH1 proteins, we performed a number of antibacterial and antifungal growth inhibition assays as described in Materials and methods. The results demonstrated that *C. michiganensis* subsp. *sepedonicus*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tabaci*, *C. coccoides* and *B. cinerea* were all affected by SN1 and PTH1, although to differing degrees (Fig. 4). Among the bacteria we tested, *C. michiganensis* subsp. *sepedonicus* was the most susceptible to both proteins and growth was completely inhibited by 14 μM of SN1 and 7 μM of PTH1. *P. syringae* pv. *syringae* and *P. syringae* pv. *tabaci* showed a low level of sensitivity to both proteins. There was no significant growth inhibition of *P. syringae* pv. *syringae* at the highest protein concentrations used in our experiments. In contrast, 14 μM of SN1 and PTH1 led to about 20% and 50% inhibition of the *P. syringae* pv. *tabaci* cells, respectively.

For the fungal pathogens, 14 μM of SN1 led to a complete inhibition of *C. coccoides* spore germination, while only about 90% inhibition of spore germination was achieved by the same concentration of PTH1 (Fig. 4) *B. cinerea* showed different responses to treatments with SN1 and PTH1. Spore germination was completely inhibited with 14 μM of SN1 whereas the same concentration of PTH1 led only to 50% inhibition (Fig. 4). Thus *C.*

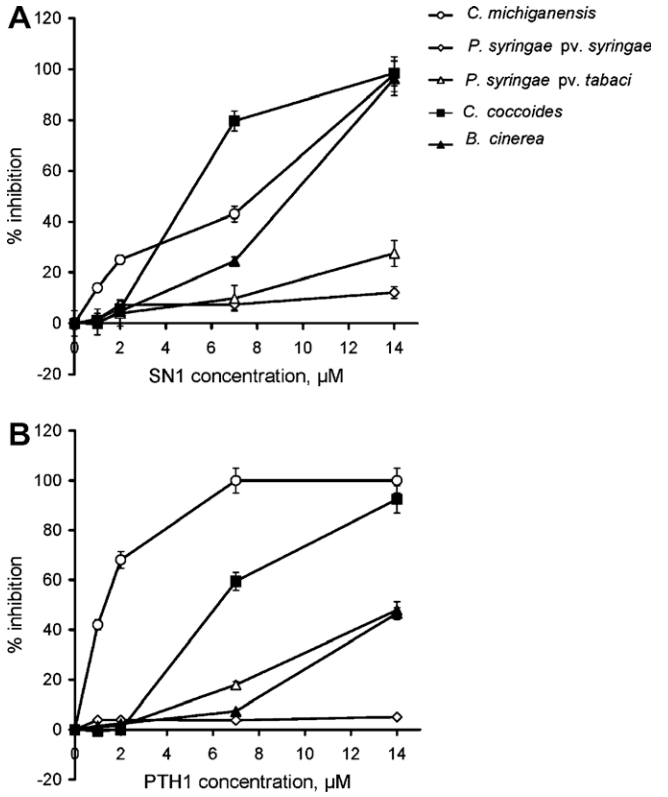

Fig. 4. The antimicrobial effect of SN1 (A) and PTH1 (B) on various phytopathogenic microorganisms.% inhibition on the *Y*-axis refers to CFU in bacteria (*C. michigenensis*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tabaci*) and spore germination in fungi (*C. coccoides*, *B. cinerea*). Each result is a mean of three replications. Values are expressed as means ± 5%. The legend on the graph refers to samples in both (A and B).

coccoides appears to be the most sensitive to action of the proteins between the fungi tested.

A synergistic effect of SN1 and PTH1 used in combination was observed against bacteria *P. syringae* pv. *syringae* and an additive effect was observed against *P. syringae* pv. *tabaci* (Fig. 5).

Aggregation of the bacterial cells was observed only for bacterial culture *C. michiganensis* subsp. *sepedonicus* upon treatment with SN1 alone as well as the combination of SN1 and PTH1 at all the concentrations tested (data not shown). In contrast, no aggregation was observed for *P. syringae* pv. *syringae* or *P. syringae* pv. *tabaci* in our experiments.

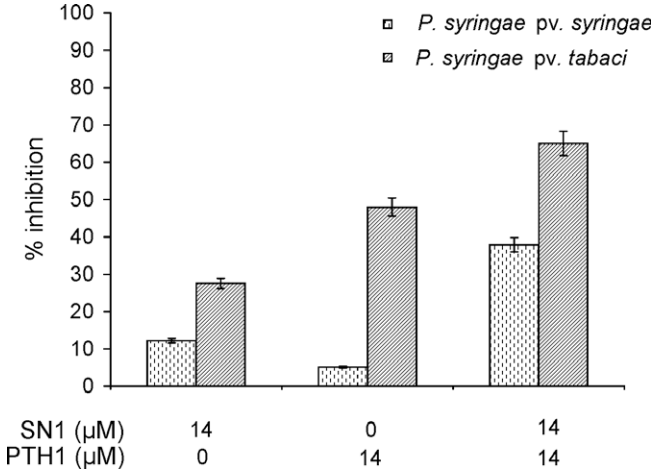

Fig. 5. Synergistic antimicrobial effect of a combination of SN1 and PTH1 against phytopathogenic microorganisms. The left bar of each treatment is *P. syringae* pv. *syringae* and the right bar is *P. syringae* pv. *tabaci*. Each result is the mean of three replications.% inhibition represents CFU. Values are expressed as ± 5%.

Discussion

In this study, we demonstrate, for the first time, the production of functionally active plant SN1 and PTH1 proteins, suitable for antimicrobial *in vitro* assays, using a prokaryotic expression system. SN1 and PTH1, naturally occurring in the potato tuber, are highly basic, cysteine-rich proteins that form six and four structure-stabilizing disulfide bridges, respectively [16,32,35]. The expression of SN1 and PTH1 in *E. coli* from the pET plasmid vector resulted in formation of IBs. Analysis of purified and solubilized IBs by gel electrophoresis showed that the target proteins were present in bacterial cells predominantly in the form of insoluble aggregates. It is well known that, in most cases, adjustment of IB washing conditions allows isolation of IBs containing more then 90% pure recombinant protein [36]. We took advantage of this approach in our study and it allowed us to successfully obtain purified preparations of SN1 and PTH1 that were used in our microbiological assays.

The presence of antibacterial and antifungal activities was demonstrated in our *in vitro* experiments for both SN1 and for PTH1 proteins expressed in *E. coli*. The overall level of those activities was similar to those of proteins obtained from the plant tissues [15,16,32]. Remarkably, the recombinant PTH1 showed a several-fold higher inhibitory activity against *C. michiganensis* subsp. *sepedonicus* as compared to that previously reported for PTH1 obtained from the plant tissues [16,32]. In previous reports, it has also been shown that the spectrum of the SN1 activity is distinct from that of PTH1. In particular, SN1 is active against both bacterial and fungal species, whereas PTH1, while showing a considerable antifungal activity, possesses little antibacterial activity [14,16,37]. However, the clearly defined difference between the spectra of antimicrobial activities of both proteins was not observed in our experiments. We found that both SN1 and PTH1 possess significant antibacterial activities against *C. michiganensis* subsp. *Sepedonicus*. However, while SN1 was active against both *C. coccoides* and *B. cinerea* fungal pathogens, PTH1 had more inhibitory activity against *C. coccoides*.

In our work we also observed a synergistic effect of SN1 and PTH1 used in combination against cultures of *P. syringae* pv. *syringae* and an additive effect against cultures of *P. syringae* pv. *tabaci*, which is fully consistent with the synergistic and additive antimicrobial effects against phytopathogens previously reported for both SN1 and PTH1 [16].

SN1 resulted in aggregation of the gram-positive bacteria *C. michiganensis* subsp. *sepedonicus* at all the concentrations tested; however, there was no correlation with the antibacterial activity of this protein *in vitro*. Our data confirm those described in previous reports with regard to snakins: snakin-1 and snakin-2 [15,16]. It is believed that the aggregation effect plays a role in the control of pathogens *in vivo*, providing a protective barrier against spreading of infectious agents through the living organism [15,38]. However, the exact mechanism of action of snakins remains unknown.

In conclusion, the recombinant plant SN1 and PTH1 proteins overexpressed in and purified from *E. coli* possessed distinct antibacterial and antifungal activities commensurate with activities of the same proteins obtained from plant tissues. The observation that the antimicrobial peptides were not toxic to the *E. coli* host used to produce the proteins may be explained by fact that the pET26b+ vector contains a tightly controlled T7*lac*promoter in BL21 (DE3) cells, where basal level transcription is low until induced by IPTG [39], and that the bacteria sequester the toxic pro-

teins into IBs where they are not functional [19]. Although we were unable to perform physical evaluations of the proteins by analytical reverse phased HPLC and mass spectrometry, the proteins were highly active biologically.

It is important to note that production of recombinant proteins in plant tissues is an expensive and time-consuming procedure, typically yielding suboptimal quantities of transgenic proteins that may not be sufficient for biological assays. In this regard, our study demonstrates the use of a less costly and technically more advantageous prokaryotic expression system for generation of cysteine-rich antimicrobial proteins that are suitable for *in vitro* biological characterization. Proteins with the best antimicrobial characteristics demonstrated in initial *in vitro* assays on a broad range of microorganisms can be selected for further evaluation *in vivo*.

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References

[1] J.M. Harvey, Reduction of losses in fresh fruits and vegetables, Ann. Rev. Phytopath. 16 (1978) 321–341.
[2] G.N. Agrios, Plant Pathology, fourth ed., Academic Press, San Diego, CA, 1997.
[3] L.S. Thomashow, D.M. Weller, Current concepts in the use of introduced bacteria for biological disease control: mechanism and antifungal metabolites, in: G. Stacey, N.T. Keen (Eds.), Plant-Microbe Interactions, vol. 1, Chapman and Hall, New York, 1996, pp. 187–235.
[4] G.D. Lyon, T. Reglinski, A.C. Newton, Novel disease control compounds: the potential to 'immunize' plants against infection, Plant Pathol. 44 (1995) 407–427.
[5] C.J. Lamb, J.A. Ryals, E.R. Ward, R.A. Dixon, Emerging strategies for enhancing crop resistance to microbial pathogens, Biotechnology 10 (1992) 1436–1445.
[6] W.F. Broekaert, B.P. Cammue, M.F. De Bolle, K. Thevissen, G.W. De Samblanx, R.W. Osborn, Antimicrobial peptides from plants, Crit. Rev. Plant Sci. 16 (1997) 297–323.
[7] M.J. Carmona, A. Molina, J.A. Fernandez, J.J. Lopez-Fando, F. Garcia-Olmedo, Expression of the alpha-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens, Plant J. 3 (1993) 457–462.
[8] Y. Huang, R.O. Nordeen, M. Di, L.D. Owens, J.H. McBeath, Expression of an engineered cecropin gene cassette in transgenic tobacco plants confers resistance to *Pseudomonas syringae* pv. *tabaci*, Phytopathology 87 (1997) 494–499.
[9] A.G. Rao, Antimicrobial peptides, Mol. Plant Microbe Interact. 8 (1995) 6–13.
[10] Y.L. Chan, V. Prasad, C. Sanjaya, K.H. Chen, P.C. Liu, M.T. Chan, C.P. Cheng, Transgenic tomato plants expressing an Arabidopsis thionin (Thi2.1) driven by fruit-inactive promoter battle against phytopathogenic attack, Planta 221 (2005) 386–393.
[11] O.L. Franco, A.M. Murad, J.R. Leite, P.A. Mendes, M.V. Prates, C. Bloch, Identification of cowpea gamma-thionin with bactericidal activity, FEBS J. 273 (2006) 3489–3497.
[12] A. Molina, F. Garcia-Olmedo, Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2, Plant J. 12 (1997) 669–675.
[13] A.G. Gao, S.M. Hakimi, C.A. Mittanck, Y. Wu, B.M. Woerner, D.M. Stark, D.M. Shah, J. Liang, C.M.T. Rommens, Fungal pathogen protection in potato by expression of plant defensin peptide, Nat. Biotechnol. 18 (2000) 1307–1310.
[14] H. Saitoh, A. Kiba, M. Nishihara, S. Yamamura, K. Suzuki, R. Terauchi, Production of antimicrobial defensin in *Nicotiana benthamiana* with a Potato Virus X vector, Mol. Plant Microbe Interact. 14 (2001) 111–115.
[15] M. Berrocal-Lobo, A. Segura, M. Moreno, G. Lopez, F. Garcia-Olmedo, A. Molina, Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection, Plant Physiol. 128 (2002) 951–961.
[16] A. Segura, M. Moreno, F. Madueno, A. Molina, F. Garcia-Olmedo, Snakin-1, a peptide from potato that is active against plant pathogens, Mol. Plant Microbe Interact. 12 (1999) 16–23.
[17] X. Yang, Y. Xiao, X. Wang, Y. Pei, Expression of a novel small antimicrobial protein from the seeds of Motherwort (*Leonurus japonicus*) confers disease resistance in tobacco, Appl. Environ. Microbiol. 73 (2007) 939–946.
[18] P. Veronese, M.T. Ruiz, M.A. Coca, A. Hernandez-Lopez, H. Lee, J.I. Ibeas, B. Damsz, J.M. Pardo, P.M. Hasegawa, R.A. Bressan, M.L. Narasimhan, In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy, Plant Physiol. 131 (2003) 1580–1590.
[19] A. Villaverde, M.M. Carrio, Protein aggregation in recombinant bacteria: biological role of inclusion bodies, Biotechnol. Lett. 25 (2003) 1385–1395.
[20] R.S. Rajan, M.E. Illing, N.F. Bence, R.R. Kopito, Specificity in intracellular protein aggregation and inclusion body formation, Proc. Natl. Acad. Sci. USA 98 (2001) 13060–13065.
[21] R. Vincentelli, S. Canaan, V. Campanacci, C. Valencia, D. Maurin, F. Frassinetti, L. Scappucini-Calvo, Y. Bourne, C. Cambillau, C. Bignon, High-throughput automated refolding screening of inclusion bodies, Protein Sci. 13 (2004) 2782–2792.
[22] M. Kęsik, V. Sączyńska, B. Szewczyk, A. Płucienniczak, Inclusion bodies from recombinant bacteria as a novel system for delivery of vaccine antigen by the oral route, Immunol. Lett. 91 (2004) 197–204.
[23] A. Middelberg, Preparative protein refolding, Trends Biotechnol. 20 (2002) 437.
[24] H.P. Sørensen, K.K. Mortensen, Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*, J. Biotechnol. 115 (2005) 113–128.
[25] M. Mujacic, K.W. Cooper, F. Baneyx, Cold-inducible cloning vectors for low-temperature protein expression in *Escherichia coli*: application to the production of a toxic and proteolytically sensitive fusion protein, Gene 238 (1999) 325–332.
[26] M. Ferrer, T.N. Chernikova, K.N. Timmis, P.N. Golyshin, Expression of a temperature-sensitive esterase in a novel chaperone-based *Escherichia coli* strain, Appl. Environ. Microbiol. 70 (2004) 4499–4504.
[27] L. Dumon-Seignovert, G. Cariot, L. Vuillard, The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3), Protein Expr. Purif. 37 (2004) 203–206.
[28] G. Lewis, I.W. Taylor, A.W. Nienow, C.J. Hewitt, The application of multi-parameter flow cytometry to the study of recombinant *Escherichia coli* batch fermentation processes, J. Ind. Microbiol. Biotechnol. 31 (2004) 311–322.
[29] F.U. Hartl, M. Hayer-Hartl, Molecular chaperones in the cytosol: from nascent chain to folded protein, Science 295 (2002) 1852–1858.
[30] R.B. Kapust, D.S. Waugh, *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused, Protein Sci. 8 (1999) 1668–1674.
[31] H.P. Sørensen, K.K. Mortensen, Advanced genetic strategies for recombinant protein expression in *Escherichia coli*, Microb. Cell Fact. 4 (2005) 1–12.
[32] M. Moreno, A. Segura, F. Garcia-Olmeda, Pseudothionin-St1, a potato peptide active against potato pathogens, Eur. J. Biochem. 223 (1994) 135–139.
[33] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[34] E.O. King, M.K. Ward, D.E. Raney, Two simple media for demonstration of pycocyanin and fluorescin, J. Lab. Clin. Med. 44 (1954) 301–307.
[35] B.P.H.J. Thomma, B.P.A. Cammue, K. Thevissen, Plant defensins, Planta 216 (2002) 193–202.
[36] J. Sambrook and D. W. Russell (2001) Molecular cloning: a laboratory manual, third ed. http://www.MolecularCloning.com, 3, 15–49.
[37] W.F. Broekaert, F.R. Terras, B.P. Cammue, R.W. Osborn, Plant defensins: novel antimicrobial peptides as components of the host defense system, Plant Physiol. 108 (1995) 1353–1358.
[38] J. Pérez-Vilar, R.L. Hill, The structure and assembly of secreted mucins, J. Biol. Chem. 274 (1999) 31751–31754.
[39] J.W. Dubendorff, F.W. Studier, Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor, J. Mol. Biol. 219 (1991) 45–59.