Antibacterial and Antifungal Activity of a Snakin-Defensin Hybrid Protein Expressed in Tobacco and Potato Plants

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Abstract: To enhance plant protection against phytopathogens, we constructed a fusion gene (SAP) for the simultaneous production of snakin-1 (SN1) and defensin-1 (PTH1) cysteine-rich antimicrobial proteins originally identified in potato. Prior to in vivo evaluation of antimicrobial activity, SAP produced in \textit{Escherichia coli} was tested in in vitro against four phytopathogenic microorganisms. SAP exhibited the highest antimicrobial activity against the bacterium \textit{Clavibacter michiganensis} subsp. \textit{sepedonicus}, which is the cause of potato ring rot disease, and the anthracnose-causing fungus \textit{Colletotrichum coccodes}, by complete inhibition of cell growth or spore germination, respectively, at a concentration of 6 \(\mu\)M. Notably, SAP showed higher inhibitory activities against \textit{Pseudomonas syringae} pv. \textit{syringae}, \textit{P. syringae} pv. \textit{tabaci} and \textit{Colletotrichum coccodes}, than individual SN1 and PTH1, whereas its effect on \textit{C. michiganensis} subsp. \textit{Sepedonicus} in \textit{in vitro} was comparable to that of PTH1 alone. Antimicrobial activity of SAP against \textit{C. coccodes} and \textit{C. michiganensis} subsp. \textit{sepedonicus} was assessed in plants on \textit{Nicotiana benthamiana} and \textit{Solanum tuberosum}, respectively, using SAP expressed from the \textit{Potato virus X}-based vectors pP2C2S and/or pGR107. Both SAP-producing plants were significantly more resistant to infection than control plants. Our results demonstrated that in vivo co-production of recombinant SN1 and PTH1 as a hybrid protein is a promising strategy for antimicrobial plant defense applications.

Keywords: Plant antimicrobial proteins, hybrid protein, PVX-based vector, anthracnose, potato ring rot disease, agroinfiltration.

INTRODUCTION

Phytopathogens attack a wide range of agriculturally important crops, causing significant yield losses. Traditional means of fighting microbial infections in agricultural crop species include application of effective microbical chemicals and/or deployment of pathogen resistant cultivars. However, the biologically dynamic nature of plant pathogens leads to the constant emergence of new virulent strains of microorganisms that are either less sensitive to certain microbical chemicals or capable of overcoming the existing resistance of plants to those pathogens.

All living organisms, including plants, have evolved diverse inducible or constitutive mechanisms to protect themselves against microbial invasion. These mechanisms in plants include production of proteins (including antimicrobial proteins (AMPs)), secondary metabolites (such as terpenes, phenolics, nitrogen- and sulfur-containing compounds, which are toxic to microbes), reactive oxygen species (such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical, which play an important role in biotic and abiotic stress responses), establishment of structural barriers (such as lignin, tannins, cellulose, and polysaccharides that prevent penetration and colonization of microorganisms) and/or a hypersensitive response that results in programmed death of the infected plant cells, thereby isolating the invading pathogens from the source of nutrients [1-3].

In recent years, AMPs have been the subject of interest as primary candidates for plant protection applications [4]. AMPs exhibit a broad range of activities against bacteria, fungi, viruses and protozoa, with low minimal inhibitory concentrations required, the occurrence of rapid microbial cell damage, and microorganisms that do not acquire resistance to AMPs due to their mechanism of action [1, 5]. A well-characterized mechanism of action of AMPs is a charged-based interaction of the AMP with phospholipids [6]. Due to the presence of several basic amino acid residues, AMPs are often positively charged at a neutral pH. Electrostatic interactions between positively-charged AMPs and negatively-charged membranes of cellular pathogens could lead to pore formation or disruption of the lipid bilayer, impairing the integrity of the plasma membrane. This unique mode of action makes the induction of resistance in the microorganism difficult because it requires dramatic changes in pathogen phospholipid membrane composition or organization [7-10]. These characteristics make the use of AMPs for developing resistant crops most appealing.
AMPs include naturally occurring prokaryotic [11-13] and eukaryotic proteins [8, 14-25] as well as engineered and synthetic proteins [8, 12, 26, 27]. Although hundreds of plant-produced AMPs have been characterized to date, only a few have been utilized commercially [28]. A comprehensive information about the known natural AMPs can be found online at http://bbcm1.univ.trieste.it/~tossi/pag1.htm [29] and http://phytamp.pfba-lab.org [30].

Recently, the activities of individual AMPs against a wide range of phytopathogens have been demonstrated in vitro as well as in vivo [18, 31-39]. In some cases, the simultaneous expression of more than one antimicrobial plant protein has been found to be necessary to achieve a significant level of plant protection [40, 41]. In addition, the use of hybrid proteins of non-plant origin such as cecropinB-thanatin and cecropinA-magainin2, cecropinA-melittin and their derivatives has recently been explored as an alternate antimicrobial protein expression strategy [5, 7, 42]. In the case of the cecropinB-thanatin hybrid protein, it possesses an improved biological activity as compared to the individual parental proteins [42]. The derivative of cecropinA-melittin hybrid showed inhibition activity against the plant-pathogenic bacteria Erwinia amylovora, Pseudomonas syringae pv. syringae and Xanthomonas vesicatoria at a concentration less than 10 μM [7].

In the present work we engineered a hybrid antimicrobial protein from components of plant origin. We fused genes encoding two cysteine-rich AMPs naturally produced in potato plants: snakin-1 (SN1) and defensin-1 (PTH1). SN1 and PTH1 possess different spectra of anti-phytopathogen activities. SN1 is active against both bacterial and fungal species, whereas PTH1 shows primarily antifungal activity [35, 43, 44]. We have also previously shown that individual SN1 and PTH1 proteins possessed significant antimicrobial activity against Clavibacter michiganensis subsp. sepedonicus, Pseudomonas syringae pv. syringae, Pseudomonas syringae pv. tabaci, Colletotrichum coccoides and Botrytis cinerea, all of which cause diseases in vegetable crops; however, in our studies, a clearly defined difference between the spectra of antimicrobial action of both proteins was not observed [31].

The aim of the present study was to develop a genetic strategy for the simultaneous production of SN1 and PTH1 in the form of self-cleavable hybrid protein (SAP) to allow formation of individual SN1 and PTH1 in plant cells. To evaluate the antimicrobial properties of SAP in vivo we used a transient-expression system based on Potato virus X (PVX)-vectors: pP2C2S and pGR107 [45, 46]. Before performing in vivo experiments, SAP was produced in and purified from Escherichia coli and tested in vitro on four species of phytopathogenic microorganisms.

**MATERIALS AND METHODS**

**Plasmid Constructions**

**Cloning of Potato sn1 and pth1 Genes**

Snakin-1 (sn1) and defensin-1 (pth1) genes were cloned from stem tissues of young potato seedlings according to standard procedures [47]. Briefly, fresh stem tissues were ground to a powder in liquid nitrogen and total RNA was extracted using a TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH). First strand cDNA was synthesized using primers SN1R and PTH1R (Table 1), respectively, and the double-stranded cDNA was amplified from the first strand cDNA using primer pairs SN1F/SN1R and PTH1F/PTH1R (Table 1), respectively. The sn1 gene was cloned into a TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) to produce pCRsn1. The pth1 gene was cloned into a plasmid vector pSKII+ (Stratagene, La Jolla, CA) at the BamHI/HindIII sites resulting in pSKpth1.

**Synthesis of FMDV 2a Protease Gene**

The coding sequence of the FMDV 2A protease was generated from the amino acid sequence of the peptide by

<table>
<thead>
<tr>
<th>Table 1. Oligonucleotide Primers Used for Cloning</th>
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<tr>
<td>Primer</td>
</tr>
<tr>
<td>SN1R</td>
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<tr>
<td>PTH1R</td>
</tr>
<tr>
<td>SN1F</td>
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<tr>
<td>PTH1F</td>
</tr>
<tr>
<td>2AF</td>
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<td>CMSIF1</td>
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<td>CMSIR1</td>
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*Restriction sites are underlined; nucleotides in italics encode the 6xHis-tag.
reverse translation [48]. A pair of complementary and partially overlapping primers, 2A2 and 2AR was designed to synthesize the gene by polymerase chain reaction (PCR) (Table 1). The synthetic 2a protease gene was then cloned into a plasmid vector pUC19 in EcoRI/BamHI sites giving rise to pUC2a, which was verified by sequence analysis.

Cloning of the Sap Gene into a PVX-Based Vector pP2C2S

The multiple cloning site (MCS) of the vector pP2C2S [45]; a gift of Dr. David Baulcombe, Sainsbury Centre, UK) was modified to incorporate additional restriction sites (not shown). The available restriction sites in the expanded MCS included: Clal, NruI, EcoRV, Ncol, BamHI, EcoRI, HindIII and Sall. To assemble the snl/2a/pth1 expression cassette, the snl gene was first moved from pCRsnl to pP2C2S at the Ncol/EcoRI sites of the expanded MCS, giving rise to pP2C2S/snl. The pth1 gene was excised from pSKpth1 and ligated to pUC2a at BamHI/HindIII sites giving rise to pUC2apth1. The 2a/pth1 insert was in turn excised from pUC2apth1 and moved to pP2C2S/snl at EcoRI/HindIII sites. The resulting construct was named pP2C2S/sap.

Construction of the pET-Based Expression Cassette

The coding region of the sap gene was amplified from plasmid pP2C2S/sap using PCR and primer pair SN12F/PTH12HisR was used to produce this construction. The PCR products were cloned into the pCR®II-TOPO®Vector (Invitrogen), resulting in recombinant plasmids pCRsap and pCRsapHis. These plasmids were subsequently digested with Ncol and HindIII and the corresponding restriction fragments were cloned into the plasmid vector pET26b(+) (Novagen, Madison, WI) at the Ncol/HindIII sites, giving rise to pET26b(+)/sap and pET26b(+)/sapHis. All constructions were verified by direct DNA sequencing.

Construction of the Binary PVX-Based Vector pGR107

The MCS of the vector pGR107 (a gift of Dr. David Baulcombe, Sainsbury Centre, UK; 46) was modified to incorporate additional restriction sites (not shown). The available restriction sites in the expanded MCS included: MluI, Call, Small, Sall, Nofi. To assemble the expression cassette, the sap gene was amplified from pP2C2S/sap using primer pair MluKozSPF and PTH1plt1R (Table 1). The sap gene was then cloned into plasmid vector pGR107 at MluI / Nofi sites giving rise to pGR107/sap.

Protein Extraction and Characterization

Protein Expression in Bacteria

E. coli strain BL21 (DE3) (Stratagene, La Jolla, CA) was used as a host for expression of the target gene. Transformation of E. coli by pET26b(+)/sap and pET26b(+)/sapHis was performed according to the manufacturer’s instructions. Protein expression, extraction, inclusion body (IB) purification, solubilization, and refolding were performed as previously described [31].

Protein Extraction from Plants

The CellLytic™P Plant Cell Lysis/Extraction Reagent (Sigma Chemical Co., Saint Louis, MO) was used to extract total plant protein from plant cells. The extraction was carried out according to the manufacturer’s instructions.

Gel Electrophoretic Characterization of Proteins

Aliquots of the SAP and SAP-His proteins were subjected to electrophoresis in a gradient Novex® Tris-Glycine Gel (10 to 20%; Invitrogen) under denaturing conditions according to the manufacturer’s instructions. The proteins were analyzed 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 microplate reader (Model 680; Bio-Rad) [49]. All the extracted proteins were stored at -20°C in 50% glycerol to prevent freeze-thaw cycles.

Protein Purification with Nickel-Nitriol triacetate 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 of Bacterial and Plant Proteins

To verify the presence of the polyhistidine-tagged SAP (SAP-His), the protein extract was subjected to Western blot analysis. Ten µl of the soluble SAP-His (2 mg/ml) was loaded on a gradient Novex® Tris-Glycine gel (10-20%) and transferred to a nitrocellulose membrane (Invitrogen) following electrophoresis. The membrane was incubated with a 1:3000 dilution of monoclonal anti-polyhistidine clone HIS-1 (mouse IgG2a isotype) antibody (Sigma) followed by a 1:5000 dilution of goat anti-mouse phosphatase-labeled antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The membrane was developed by utilizing the BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Inc.).

To confirm the production of SAP in plants, the total plant protein fraction was subjected to Western blot analysis. Twenty µl of the total plant protein fraction was loaded on a gradient Novex® Tris-Glycine Gel (10-20%) and transferred to a nitrocellulose membrane (Invitrogen). The membrane was incubated with a 1:200 dilution of polyclonal anti-SN1PTPH1 (rabbit IgG (H+L) isotype) antibody (Cocalico Biologicals, Inc., Reamstown, PA) followed by a 1:5000 dilution of goat anti-rabbit phosphatase-labeled antibody (Kirkegaard & Perry Laboratories, Inc.)

Infection of Plants

Preparation and Delivery of Infectious Transcripts

pP2C2S/sap and pP2C2S, linearized at the SpeI site, were used as templates for the synthesis of capped transcripts in 20 µL reaction using T7 mMessage mMachine kit (Ambion, Inc., Austin, TX). The transcripts were diluted twice with 50 mM K2HPO4 (pH 7.2) and rubbed onto leaves (rub-infection) of experimental plants using carborundum. Three leaves were infected for each plant. The plants of Nicotiana benthamiana were infected at 4-5-leaf stage, Solanum tuberosum – at 3 weeks age. All rub-infected plants were grown in a green house at 27±2°C under 18-h/6-h light/dark photoperiod for 10 (N. benthamiana) or 14 (S. tuberosum) days until inoculation by pathogenic microorganisms.
**Preparation of Agrobacterium Competent Cells**

Five ml of LBG (LB medium supplemented with 0.4% glucose) with tetracycline (10μg/ml, final concentration) was inoculated by *Agrobacterium tumefaciens* strain GV3101 containing helper plasmid pJIC SA_Rep and grown at 28°C overnight at 250rpm. Five ml of overnight culture was added to 50 ml LBG (with tetracycline) and agitated at 250rpm at 28°C until the culture density reached OD600 of 0.5 to 1.0 (~8hrs). After chilling for 10 min on ice the culture was centrifuged at 4000 rpm for 20 min at 4°C. The pellet was resuspended in 1 ml of 20mM of ice-cold CaCl2 solution and 0.1-ml aliquots of cell suspension were dispensed into pre-chilled Eppendorf tubes and stored at -80°C.

**Introduction of pGR107/Sap and pGDp19 into A. Tumefaciens**

One μg of either pGR107/sap or pGDp19 [expressing a viral silencing suppressor protein, 50] plasmid DNA was added to the thawed competent cells and frozen in liquid nitrogen for 5 min. Heat shock was performed at 37°C for 25 min. The cells were supplied with 1 ml of LBG medium containing tetracycline followed by incubation at 28°C for 3 hours with shaking at 150 rpm. The tubes were centrifuged for 30 s at maximum speed and the pellets were resuspended in 50 μl of LBG medium and spread on an LBG agar plates containing tetracycline (10μg/ml) and kanamycin (50μg/ml). The plates were incubated at 28°C for 5 days. The positive agrobacterium colonies were reconfirmed by PCR before agroinfiltration.

**Agroinfiltration**

Five ml of LBG medium containing tetracycline (10μg/ml, final concentration) and kanamycin (50μg/ml, final concentration) was inoculated with transformed agrobacterium cells and incubated overnight at 28°C at 250 rpm. The cultures were centrifuged at 4000 rpm at 25°C for 20 min and the bacterial pellets were gently re-suspended in infiltration medium (10 mM MgCl2, 10 mM MES pH5.7, 150μM acetylsyringone) to an OD600 of 0.5-1.0 and left at ambient temperature for 3 hours. Prior to the infiltration procedure, one part of the silencing suppressor pGDp19 [50] and 9 parts of pGR107/sap were mixed. *N. benthamiana* plants at leaf stage 4-5 were used for infiltration. Leaves were agroinfiltrated at the abaxial side with the bacterial suspension using a 1-ml needle-less syringe. Two leaves were infiltrated per plant. Infiltrated plants were maintained in a green house at 27±2°C under 14-h/10-h light/dark photoperiod for 7 days until inoculation by *C. coccoides*.

**Studies with Microorganisms**

**Microorganisms**

The list of the microorganisms used in experiments is displayed in (Table 2).

* C. *michiganensis* subs. *sepedonicus* (hereinafter referred to as *C. michiganensis*), a Gram-positive bacterium, causing potato ring rot disease, was grown on nutrient-broth yeast extract agar (NBY) [8.0g of nutrient broth (Difco, Detroit), 2.0 g of yeast extract, 2.0 g of K2HPO4, 0.5 g of KH2PO4, 2.5 g of glucose and 15.0 g of Bacto agar per 1 liter of H2O; after autoclaving, 1.0 ml of a sterile solution of 1M MgSO4 was added]. After inoculation of solid medium with the bacterium, the plates were incubated for five days at 28°C. For antibacterial assays the colonies from the plate were transferred into a culture tube containing NBY broth and grown at 28°C with vigorous shaking at 250 rpm for 168 hours.

* P. syringae* pv. *syringae* and *P. syringae* pv. *tabaci*, Gram-negative bacteria, were grown on KB medium [20.0 g of Proteose peptone #3 (Difco, Detroit, MI), 1.5 g K2HPO4, 15.0 ml glycerol, 15.0g Bacto agar per liter of H2O; after autoclaving, 6.0 ml of a sterile solution of 1M MgSO4 was added] [51]. The culture plates were incubated for two days at 28°C. For antibacterial assays 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* causing anthracnose of various plant species was routinely cultured on Potato Dextrose Agar (PDA) (Difco) plates for approximately 14 days at room temperature. For antifungal assays in *in vitro* and *in vivo*, the spores were collected and suspended in Potato Dextrose Broth (PDB) (Sigma-Aldrich, Inc., St. Louis, MO) or distilled sterile water, respectively. Spore concentrations were determined using hemacytometer and light microscope (Zeiss Axioskop 2).

<table>
<thead>
<tr>
<th>Phytopathogens</th>
<th>Plants</th>
<th>Constructions and Their Delivery into the Plants</th>
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<tbody>
<tr>
<td><em>Colletotrichum coccoides</em></td>
<td><em>Nicotiana benthamiana</em></td>
<td><em>pGR107</em> or <em>pP2C2S</em></td>
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<tr>
<td><em>Pseudomonas syringae</em> pv. <em>syringae</em> 61</td>
<td><em>Solanum tuberosum</em></td>
<td><em>pP2C2S</em> or <em>pP2C2S/sap</em></td>
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<tr>
<td><em>Pseudomonas syringae</em> pv. <em>tabaci</em> 11528 Race 0</td>
<td>n/a</td>
<td><em>pP2C2S</em> or <em>pP2C2S/sap</em></td>
</tr>
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</table>
Antibacterial Assays

In Vitro Assay

The antibacterial activity of SAP protein recovered from IBs was determined against C. michiganensis, P. syringae pv. syringae, P. syringae pv. tabaci as previously described [31], using 0, 0.6, 1.2, 3.0 and 6.0 μM in 1 ml of total reaction volume. The bacterial concentration in the beginning of the experiment was 1×10^8 CFU per ml for C. michiganensis, 4×10^7 CFU per ml for P. syringae pv. syringae and 8×10^7 CFU per ml for P. syringae pv. tabaci. The reaction mixtures were incubated at 28°C with continuous aeration at 250 rpm for 192 and 17 hours for C. michiganensis and pseudomonades, respectively. Following the incubation, 100 μl aliquots of protein-treated bacterial cultures were serially diluted in sterile water (from 10^-2 to 10^-8) and 25 μl of diluted bacterial suspensions were plated onto the appropriate solid medium. The plates were incubated at 28°C for 5 and 2 days for C. michiganensis and pseudomonades, respectively and examined for bacterial growth by counting CFU. Each protein concentration was determined by counting germinating and non-germinating spores, as previously described [31], using 0, 0.6, 1.2, 3.0 and 6.0 μM in 1 ml of total reaction volume. The bacterial concentration in the beginning of the experiment was 1×10^8 CFU per ml for C. michiganensis, 4×10^7 CFU per ml for P. syringae pv. syringae and 8×10^7 CFU per ml for P. syringae pv. tabaci. The reaction mixtures were incubated at 28°C with continuous aeration at 250 rpm for 192 and 17 hours for C. michiganensis and pseudomonades, respectively. Following the incubation, 100 μl aliquots of protein-treated bacterial cultures were serially diluted in sterile water (from 10^-2 to 10^-8) and 25 μl of diluted bacterial suspensions were plated onto the appropriate solid medium. The plates were incubated at 28°C for 5 and 2 days for C. michiganensis and pseudomonades, respectively and examined for bacterial growth by counting CFU. Each protein concentration was analyzed in triple replication.

In Vivo Assay

Transcript-infected plants of Solanum tuberosum (red-skinned variety) were inoculated by C. michiganensis in two different procedures. The first procedure involved injection of the plant stems with C. michiganensis at a single site with 100 μL of bacterial suspension (10^8 CFU/ml) in sterile distilled water. The second procedure involved trimming of the plant roots to remove about 1/3 of root mass and their distillation with 100 μl aliquots of protein-fungus mixture was applied on the surface of a hemacytometer, which was placed into a humidifying chamber (Petri dish with wet filter paper). The inhibitory activity of SAP on spore germination was determined after 12 hours of incubation at 28ºC by visualization with light microscopy.

Antifungal Assays

In Vitro Assay

The antifungal activity of SAP against C. coccoides was determined by counting germinating and non-germinating protein-treated fungal spores, as previously described [31], using a concentration of SAP protein adjusted by PDB so that final concentrations were 0 (control), 0.6, 1.2, 3.0 and 6.0 μM. Each antifungal assay was performed in three replicates. The total volume of protein-fungus mixture was 50μl. Twenty five μl of each mixture was applied on the surface of a hemacytometer, which was placed into a humidifying chamber (Petri dish with wet filter paper). The inhibitory activity of SAP on spore germination was determined after 12 hours of incubation at 28°C by visualization with light microscopy.

In Vivo Assay

N. benthamiana plants were inoculated with a suspension of 10^6 conidia ml^-1 of C. coccoides in sterile distilled water by spraying until the fluid runoff. The plants were enclosed in a plastic box, incubated at 27±2°C under 14-h/10-h light/dark photoperiod, and monitored daily for the disease symptoms. Each inoculation experiment was repeated four times.

Light microscopy. Sections of the tobacco leaves containing infection sites were excised and placed on a glass slide with several drops of lactophenol cotton blue solution and heated over a flame for approximately 10s. Stained leaf pieces were examined by light microscopy (Zeiss, Axioskop 2).

Molecular Analysis of the Plants Expressing the Sap Gene

RT-PCR for Detection of Sap Gene

Total cellular RNA was extracted using TRI Reagent (Molecular Research Center, Inc.) from systemically infected leaves of tobacco and potato plants one/two weeks after infection by PVX transcripts, respectively. The RT-PCR analysis was carried out using Titan One Tube RT-PCR System (Roche, Germany) according to the manufacturer’s instructions with the primer pair SN1F/PTH1R at concentration 20 pmol, annealing temperature 52°C and elongation time of 2 min. For RT-PCR, 35 cycles were conducted in a GeneAmp®System 9700 (Applied Biosystems, US) with AMV reverse transcriptase for the first strand cDNA synthesis and the Expand High Fidelity enzyme blend consisting of Taq DNA polymerase and Tgo DNA polymerase for amplification of cDNA by PCR. The PCR fragments were fractionated on a 1.0% agarose gel.

Detection of C. Michiganensis in Potato Tubers by PCR Analysis

The samples of DNA were extracted from the potato tubers 40 days after inoculation by C. michiganensis using Plant DNAzol®Reagent (Invitrogen) [52]. C. michiganensis subsp. sepedonicus—specific DNA sequence was amplified by primer pair CMSIF1/CMSIR1 ([53]; Table 1). These primer pairs were designed on the sequence of the 1.3-kb insertion element IS1121, a highly repeated segment of DNA that is present in the chromosome of C. michiganensis subsp. sepedonicus [54, 55]. For PCR amplification, 35 cycles were conducted in a GeneAmp®System 9700 (Applied Biosystems, US) with AmpliTaq polymerase. The following PCR parameters were used: denaturation at 94°C for 1 min (5 min for the first cycle), annealing for 2 min at 34°C and extension for 3 min (10 min in the final cycle) at 72°C. Formamide (final concentration, 3%) was added to the reaction mixture. The PCR fragments were fractionated on a 1.0% agarose gel.

RESULTS

Expression of Sap in a Prokaryotic Expression System

To obtain a purified recombinant SAP protein, we positioned the sap gene expression cassette in the pET26b(+) vector under the control of a bacteriophage T7 promoter (Fig. 1A). The core of the expression cassette was a single open reading frame encoding the hybrid SAP protein. To allow formation of individual SN1 and PTH1 proteins, the autocatalytic cleavage site of the foot-and-mouth disease virus (FMDV) 2A protease oligopeptide [48, 56] was incorporated between sn1 and pth1 sequences of the hybrid gene. Analysis of the total and soluble fractions of bacterially-expressed SAP protein by gel electrophoresis demonstrated that SAP was localized in the insoluble fraction containing inclusion bodies (IBs) (Fig. 2A). Our attempts to express the protein at a lower temperature (25°C)
to avoid IB formation or extraction of the protein from IBs using sonication or solubilization with 8M urea failed (data not shown). After solubilization, IBs were analyzed by gel electrophoresis. The size of the protein recovered from IBs was consistent with the predicted molecular mass of SAP of approximately 14 kDa. Despite of our expectation of generating two individual SN1 and PTH1 proteins from the hybrid, no visible cleavage products were observed (Fig. 2B).

Another variant of SAP containing a carboxy-terminal six histidine (6His) tag (SAPHis) was generated in parallel to allow affinity purification of the protein. To verify the presence of the polyhistidine tag in SAP this protein was subjected to Western blot analysis using a monoclonal anti-polyhistidine antibody. The results confirmed the presence of polyhistidine in the expressed protein (data are not shown). However, our attempts to purify SAPHis on Ni-NTA resin after IB solubilization and protein refolding were unsuccessful, most likely due to some conformational alterations in this protein following it’s refolding. These alterations could result in the internalization of the 6His tag inside the protein structure rendering it inaccessible for binding to the resin. For this reason the SAP variant without polyhistidine was used for all microbiological assays of this study.

Analysis of the Antimicrobial Activity of SAP in in Vitro

To assess the antimicrobial activity of the SAP protein, we performed a number of antibacterial and antifungal growth inhibition assays as described in Materials and Methods. The growth of C. michiganensis, P. syringae pv. syringae, P. syringae pv. tabaci and spore germination of C. coccoides were affected by SAP (Fig. 3). Among the tested bacteria C. michiganensis was the most susceptible to the hybrid protein as its growth was completely inhibited by SAP at a concentration of 6 μM. The pseudomonads showed
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a less sensitivity to SAP where 6 µM of SAP led to about 20% and 30% inhibition of growth of *P. syringae* pv. *syringae* and *P. syringae* pv. *tabaci*, respectively. Antifungal activity of SAP revealed that 6 µM of SAP led to a complete inhibition of the spore germination of *C. coccoides* (Fig. 3).

Comparative analysis of activities of individual SN1 and PTH1 proteins [31] with the hybrid protein is represented in (Fig. 4). The effect of SAP on growth of *C. michiganensis* was comparable to the effect of PTH1 alone, suggesting that the activity of the hybrid protein, in this case, was primarily determined by PTH1 component of the hybrid; however, aggregation of the bacterial cells in cultures of *C. michiganensis* by SAP was similar to that observed with SN1 alone (data not shown) [31], indicating that the SN1 protein was active in the hybrid protein. In contrast, an additive effect of SAP inhibitory activity on growth was observed against *P. syringae* pv. *syringae*, *P. syringae* pv. *tabaci* and *C. coccoides*.

Microorganisms that were shown in *in vitro* experiments to exhibit the highest sensitivity to the hybrid SAP protein at the lowest concentrations were chosen for testing *in vivo*. They were the bacterium *C. michiganensis* and the fungus *C. coccoides*.

Expression of the Sap Gene in *N. Benthamiana* and Analysis of Antifungal Activity of SAP Protein Against *C. Coccoides* in *In Vivo*

For *in vivo* assays, the sap gene expression cassette was engineered into the PVX-based vector pP2C2S [45] and binary PVX-based vector pGR107 [46], under the control of bacteriophage T7 and *Cauliflower mosaic virus* 35S promoters, respectively (Fig. 1B, C), and delivered into plants as described (Materials and Methods). The stability of the engineered sap gene within both PVX-based vectors in tobacco plants was confirmed by RT-PCR assays performed on RNA samples isolated from systemically infected leaves on 9/7 days after transcript infection/agroinfiltration, respectively (Fig. 5A). Expression of the sap gene in plant tissues was confirmed by Western blot analysis in the case of agroinfiltration with the pP2C2S/sap vector (Fig. 5B), whereas in case of transcript infection with the pP2C2S/sap vector no detectable level of SAP protein in plant cells was observed (data not shown). Moreover, the employed expression strategy was expected to generate two individual antimicrobial proteins SN1 and PTH1 from the hybrid in *in vivo* but no visible cleavage products were detected (Fig. 5B), and similar to what was observed in *E. coli* (Fig. 2B).

The following treatments for antifungal assays were used: 1) “control-mock” (plant + *C. coccoides*); 2) “empty-PVX” (plant + pP2C2S or pGR107 + *C. coccoides*) and 3) “PVX/sap” (plants + pP2C2S/sap or pGR107/sap + *C. coccoides*). Anthracnose symptoms appeared in all...
treatments at 4 days post inoculation (dpi) by *C. coccoides* as small dry papery spots, regardless of the type of vector used for infection (Fig. 6A-C). These spots were enlarged to 4-6 mm in diameter at 8 dpi in the "control-mock" and "empty-PVX" treatments, whereas in plants expressing *sap* gene ("PVX/sap" group) the size of the spots did not exceed from 1 mm in diameter and remained unchanged for the duration of the experiment (18 dpi). Lesion zones were also observed on stems of the infected plants for all experimental groups (Fig. 6D-F), although the amount and the size of those lesions varied. In "control-mock" and "empty-PVX" groups the number of the stem lesions was 12-15 per plant with zone sizes ranging from 5 to 7 mm in diameter, while the stems of *sap*-expressing plants displayed only 3-5 lesions, ranging from 1 to 2 mm in diameter. Representative plants from each treatment are shown in (Fig. 6G-I). Plants expressing *sap* displayed higher levels of resistance to fungal infection than the plants in the control groups ("control-mock" and "empty-PVX"). The most severe symptoms were observed in plants of "empty-PVX" group (Fig. 6H) that developed coalescing lesions leading to complete death of the affected leaves. *C. coccoides* was isolated from the surface of the infected leaves of the *N. benthamiana* and the re-isolated fungus appeared identical to that of the original culture used for inoculation. Conidia from the re-isolated fungus were also able to infect tobacco seedlings.

Examination of conidia of *C. coccoides* was performed after staining of tobacco leaf segments with lactophenol cotton blue solution (Fig. 7). Germination of conidia and appearance of appressoria were detected within 24h after inoculation (Fig. 7A,B) on all plants. However, on plants expressing *sap* ("PVX/sap"), only single conidia germinated, whereas the majority of the conidia did not germinate and were localized along the plant cell wall (Fig. 7C). In the same group ("PVX/sap"), appressorium formation was observed only at few instances. Development of acervuli was detected in all experimental groups on the 5th dpi (Fig. 7D).

**Expression of the *Sap* Gene in Plants of *S. Tuberosum* and Analysis of Antibacterial Activity of SAP Protein Against *C. Michiganensis in vivo***

For *in vivo* experiments with potato plants we utilized the same *sap* gene expression cassette in the context of the PVX-based vector pP2C2S as for experiments with tobacco plants described above (Fig. 1B). We attempted several times unsuccessfully to perform agroinfiltration with the pGR107/sap on potato leaves. Due to technical difficulties, we proceeded further with the pP2C2S/sap experiments. The stability of the *sap* gene in pP2C2S in potato plants was confirmed by RT-PCR assays using RNA samples isolated from systemically infected leaves and tubers 2 and 8 weeks after transcript inoculation, respectively (Fig. 8A,B). However, as in the case of tobacco plants infected by pP2C2S, no detectable level of SAP protein in potato plants was observed (data not shown).

The following experimental groups for antibacterial assays were used: 1) "control-mock" (plant + *C. michiganensis*); 2) "empty-pP2C2S" (plant + pP2C2S + *C. michiganensis*) and 3) "pP2C2S/sap" (plant + pP2C2S/sap + *C. michiganensis*).
Fig. (6). Anthracnose symptoms on tobacco plants inoculated by *C. coccoides*. Fully expanded leaves of “control-mock” (A), “empty-pP2C2S” (B) and “pP2C2S/sap” (C) groups (9th day after fungal inoculation). The fragments of the tobacco stems of “control-mock” (D), “empty-pP2C2S” (E) and “pP2C2S/sap” (F) groups (12th day after fungal inoculation). Representative tobacco plants from “control-mock” (G), “empty-pP2C2S” (H) and “pP2C2S/sap” (I) groups (12th day after fungal inoculation).

Fig. (7). Sections of the tobacco leaf surfaces stained with lactophenol cotton blue solution. Germination of conidia (C) of *C. coccoides* with appressorium (Ap) formation in “control-mock” (A) and “empty-pGR107” (B) groups (2nd dpi). C: specific localization of fungal conidia along the plant cell wall (CW) in “pP2C2S/sap” group (2nd dpi). D: Acervulus (A) development in “control-mock” group (5th dpi).
Symptoms of potato ring rot disease were observed on potato plants in “control-mock” and “empty-pP2C2S” groups (Fig. 9A-C) whereas no notable disease symptoms appeared on plants expressing sap (“pP2C2S/sap”) (Fig. 9H). Bacterium inoculation techniques of the plants did not affect the infection rate or appearance of symptoms (Experimental Procedures). Above-ground symptoms were observed after approximately 3 weeks post-bacterial inoculation. The initial foliar symptoms included: wilting, yellowing at the leaf margins followed by leaf rolling (Fig. 9B) and subsequent necrosis of terminal leaves and stems (Fig. 9C). In most cases the above-ground symptoms were limited to two stems in a hill while the rest of the stems appeared normal. Furthermore, the brown necrotic spots were detected on the stems of the plants in “empty-pP2C2S” group (Fig. 9C).

Tuber symptoms appeared later and were clearly visible after 6 weeks post-bacterial inoculation in the “control-mock” group (Fig. 9D,E), whereas no tuber symptoms in other experimental groups (“empty-pP2C2S” and “pP2C2S/sap”) were observed (data not shown). The external tuber symptoms included the appearance of yellowish zones on the surface of the tubers covered with mucus (Fig. 9D). The internal tuber symptoms involved changes in the color of vascular ring from cream to brown, development of significant creamy bacterial ooze in place of infection, and disintegration of the inner part of the tubers except its outer shell (Fig. 9E).

To test the presence of C. michiganensis in potato tubers, a detection procedure based on PCR amplification of C. michiganensis subsp. sepedonicus -specific DNA was employed [54, 55]. No C. michiganensis subsp. sepedonicus –specific amplicons were detected when DNA samples isolated from tubers expressing the sap gene were used as a template, while such amplicons were detected from samples in “control-mock” and “empty-pP2C2S” groups (Fig. 8C).

A sample of infected potato plants is shown in (Fig. 9F-H). Plants expressing sap (“pP2C2S/sap” group) (Fig. 9H) exhibited a significantly higher level of resistance to bacterial infection as compared to the plants from “control-mock” and “empty-pP2C2S” groups (Fig. 9F-G) and were indistinguishable from uninfected healthy plants (Fig. 9I).

DISCUSSION

In this work, we constructed a hybrid protein SAP consisting of two mature antimicrobial proteins SN1 and PTH1 of plant origin linked by autocatalytic cleavage site (FMDV 2A protease) and demonstrated its antibacterial and antifungal activities against phytopathogens in in vitro and in vivo.

The successful production of individual SN1 and PTH1 for in vitro experiments using a pET-expression system was demonstrated previously in our laboratory [31]. In the present study, we used the same prokaryotic expression system to produce a hybrid protein. In spite of the presence in the pET-vector of an N-terminal pelB signal sequence fused to the expressed protein, which should facilitate protein localization to the periplasm and reduce or eliminate IB formation, electrophoretic analysis of the total and soluble fractions of bacterially-expressed SAP revealed that the hybrid protein was localized in the form of insoluble aggregates (e.g. IBs) (Fig. 2A). It has been reported that adjustment of the IB washing condition allows isolation of IBs containing more than 90% of pure recombinant protein [57]. We used the advantage of IB formation and successfully obtained a purified SAP protein for microbiological assays.

In our experiments, we expected to generate two individual proteins SN1 and PTH1 from the hybrid, but no visible cleavage products were detected both in prokaryotic and eukaryotic expression systems. The reason for the inability of the FMDV 2A oligopeptide to mediate cleavage in a prokaryotic system is not clear. Although the complete FMDV 2A protease was reported not to function in eukaryotes while it does function in eukaryotes [58], the 2A oligopeptide is predicted to function autocatalytically and may not require host cell proteases for cleavage [56, 59, 60].

In vitro experiments demonstrated that antimicrobial activity of the hybrid protein against P. syringae pv. syringae, P. syringae pv. tabaci and C. coccoides was higher than the activity of individual SN1 and PTH1 proteins [31] (Fig. 4). While SAP completely inhibited spore germination of C. coccoides at 6µM, the same effect for individual SN1 and PTH1 was achieved only at 14µM. A clear additive effect of SN1 and PTH1 proteins in the form of a hybrid was also demonstrated for pseudomonads: a 6µM of SAP led to
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Fig. (9). Foliar and tuber symptoms of potato ring rot disease, caused by C. michiganensis. Above-ground symptoms of potato ring rot disease on plants of “control-mock” (A-B) and “empty-pP2C2S” groups (C) (37/28 dpi, respectively; the plants were infected by root trimming). Surface (D) and cross section (E) of the potato infected tuber (“control-mock” group, 46th dpi, plants were inoculated by stem injection). Representative potato plants from “control-mock” (F), “empty-pP2C2S” (G), “pP2C2S/sap” (H) groups (37th dpi, plants were infected by root trimming). I: healthy plant (without any treatment).

about 25% and 35% inhibition of P. syringae pv. syringae and P. syringae pv. tabaci, respectively, whereas individual SN1 and PTH1 at concentration of 6μM each inhibited growth of pathogen by approximately 10% [31]. The effect of SAP on growth of C. michiganensis was comparable to the effect of PTH1 alone; however, aggregation of the bacterial cells in cultures of C. michiganensis by SAP was similar to that observed with SN1 alone [31], indicating that, despite the observation that the hybrid protein was not cleaved at the 2A oligopeptide cleavage site, the SN1 protein retained its activity in the hybrid protein.

Despite the absence of expected SAP self-cleavage in plant tissues and detectable level of SAP protein in transcript-inoculated tobacco and potato plants (“pP2C2S/sap” groups), our in vivo experiments demonstrated a clear antimicrobial effect of SAP against C. coccoides and C. michiganensis (Fig. 6 – Fig. 9). We suggest that the presence of the linker (FMDV 2A region) in SAP protein does not affect folding of SN1 and PTH1 components of the hybrid, which allows them to remain functionally active in the hybrid protein. The inability of 2A protein to undergo self-cleavage in plant cells in our in vivo experiments remains unclear, though, according to the literature data, the 2A protein has been used for a variety of biotechnological purposes and its activity has been examined in a wide range of heterologous protein contexts, so it should be active in all eukaryotic systems [48, 58-60].

The absence of a detectable level of SAP protein in tobacco and potato plants infected by vector pP2C2S carrying sap gene indicates a low level of sap gene expression or protein degradation. Low levels of gene expression may be due to activation of a post-transcriptional gene silencing (PTGS) mechanism whereby homology between nucleotide sequences of the sap gene on the virus-based vector and a gene/genes on the chromosome of host-plant results in degradation of viral RNA transcripts [61, 62]. This explanation is based on our supposition that tobacco may produce antimicrobial proteins in composition to SN1 and PTH1 to those that are present in potato, which are both members of the Solanaceae. In contrast, the expression of
The hybrid protein showed antibacterial properties in in vivo as well. No visible above-ground or tuber symptoms of the potato ring rot disease, caused by bacteria C. michiganensis, were observed in potato plants expressing sap (Fig. 9). Though, there were also no tuber symptoms on the plants expressing the “empty-p2P2C2S” cassette, but the foliar symptoms in this case were clearly visible. Both above-ground and tuber symptoms were observed in “control-mock” group. According to the literature, symptoms in infected tubers may or may not develop after 2-3 months of infected tubers storage [67]. Moreover, foliar and tuber symptoms of the bacterial ring rot disease vary depending on the potato cultivar, while most plants rarely exhibit any symptoms upon infection [68]. PCR analysis of DNA isolated from potato tubers showed the absence of C. michiganensis subsp. sepedonicus-specific DNA in plants expressing sap gene, but it was detected in plants without sap expression (Fig. 8). The hybrid SAP protein may impede the spread of bacterial cells from inoculation sites and prevent systemic infection by causing rapid aggregation of bacterial cells. We previously demonstrated the ability of the SN1 protein to aggregate cells of C. michiganensis in in vitro [31, 35].

In summary, our study showed: a) the ability to obtain a functionally active SAP protein from purified IBs, suitable for in vitro biological characterization; b) the higher effectiveness of the hybrid protein against the majority of the tested microorganisms in in vitro as compared with the activity of individual SN1 and PTH1 proteins, and c) the plant protective properties of SAP protein against anthracnose and potato ring rot disease.

The findings from the present work suggest that in vivo co-production of recombinant SN1 and PTH1 in the form of hybrid protein is a promising strategy for antimicrobial plant defense applications.

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**REFERENCES**

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