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 *Escherichia coli* was tested in *in vitro* against four phytopathogenic microorganisms. SAP exhibited the highest antimicrobial activity against the bacterium *Clavibacter michiganensis* subsp. *sepedonicus,* which is the cause of potato ring rot disease, and the anthracnose-causing fungus *Colletotrichum coccoides,* by complete inhibition of cell growth or spore germination, respectively, at a concentration of 6 μM. Notably, SAP showed higher inhibitory activities against *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tabaci* and *Colletotrichum coccoides*, than individual SN1 and PTH1, whereas its effect on *C. michiganensis* subsp. *Sepedonicus in in vitro* was comparable to that of PTH1 alone. Antimicrobial activity of SAP against *C. coccoides* and *C. michiganensis* subsp. *sepedonicus* was assessed in plants on *Nicotiana benthamiana* and *Solanum tuberosum,* respectively, using SAP expressed from the *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 microbicidal 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 microbicidal 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.

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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 *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 10μM [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 *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 *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 pCR*sn1*. The *pth1* gene was cloned into a plasmid vector pSKII(+) (Stratagene, La Jolla, CA) at the *Bam*HI/*Hin*dIII sites resulting in pSK*pth1*.

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 1. Oligonucleotide Primers Used for Cloning

Primer	Nucleotide Sequence (5' → 3')[a]	Restriction Sites
SN1R	GGAATTCAGGGCATTTAGACTTGC	*Eco*RI
PTH1R	CCCAAGCTTAGCATGGCTTAGTGCA	*Hin*dIII
SN1F	CTTAGCCATGGTGAAGTTATTTCTATTAAC	*Nco*I
PTH1F	CGGGATCCAGACATTGCGAGTCGTTG	*Bam*HI
2AF	GGAATTCAACTTCGATCTTTTGAAGCTAGCAGGAGATGTTGA	*Eco*RI
2AR	CTGGATCCTGGACCTGGATTAGATTCAACATCTCCTGCTAGC	*Bam*HI
SN12F	CTTAGCCATGGCTGGTTCAAATTTTTGTG	*Nco*I
PTH12R	TAAGAAGCTTTTATCAGCATGGCTTAGTGCAAAAGCA	*Hin*dIII
PTH12HisR	GCCGCAAGCTTTTATCA*GTGATGGTGATGGTGATG*GC	*Hin*dIII
MluKozSPF	CCAGACGCGTCCACCATGGTGAAGTTATTTCTATTA	*Mlu*I
PTH1plt1R	GCGGCCGCTTATCAGCATGGCTTAGTGCAAAA	*Not*I
CMSIF1	TGTACTCGGCCATGACGTTGG	-
CMSIR1	TACTGGGTCATGACGTTGGT	-

[a]Restriction sites are underlined; nucleotides in italics encode the 6xHis-tag.

reverse translation [48]. A pair of complementary and partially overlapping primers, 2AF 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 *Eco*RI/*Bam*HI sites giving rise to pUC*2a,* 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: *Cla*I , *Nru*I, *Eco*RV, *Nco*I, *Bam*HI, *Eco*RI, *Hin*dIII and *Sal*I. To assemble the *sn1/2a/pth1* expression cassette, the *sn1* gene was first moved from pCR*sn1* to pP2C2S at the *Nco*I/*Eco*RI sites of the expanded MCS, giving rise to pP2C2S/*sn1*. The *pth1* gene was excised from pSK*pth1* and ligated to pUC*2a* at *Bam*HI/*Hin*dIII sites giving rise to pUC*2apth1*. The *2a/pth1* insert was in turn excised from pUC*2apth1* and moved to pP2C2S/*sn1* at *Eco*RI/*Hin*dIII 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/ PTH12R (Table **1**), corresponding to the N- and C-termini of the SAP protein. A carboxy-terminal 6×His-tagged protein was designed to facilitate purification using a Ni-NTA resin. The 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 pCR*sap* and pCR*sapHis*. These plasmids were subsequently digested with *Nco*I and *Hin*dIII and the corresponding restriction fragments were cloned into the plasmid vector pET26b(+) (Novagen, Madison, WI) at the *Nco*I/*Hin*dIII 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: *Mlu*I, *Cal*I , *Sma*lI, *Sal*I, *Not*I. 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 *Mlu*I / *Not*I 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-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 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-SN1PTH1 (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 *Spe*I 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 K_2HPO_4 (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 OD_{600} 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 $CaCl_2$ 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 $MgCl_2$, 10 mM MES pH5.7, 150μM acetosyringone) to an OD_{600} 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 K_2HPO_4, 0.5 g of KH_2PO_4, 2.5 g of glucose and 15.0 g of Bacto agar per 1 liter of H_2O; after autoclaving, 1.0 ml of a sterile solution of 1M $MgSO_4$ was added]. After inoculation of solid medium with the bacterium, the plates were incubated for five days at 28ºC. For antibacterial assays the colonies from the plate were transferred into a culture tube containing NBY broth and grown at 28ºC 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 K_2HPO_4, 15.0 ml glycerol, 15.0g Bacto agar per liter H_2O; after autoclaving, 6.0 ml of a sterile solution of 1M $MgSO_4$ was added] [51]. The culture plates were incubated for two days at 28ºC. For antibacterial assays the colonies from the plate were transferred into a tube containing KB broth and grown at 28ºC with shaking (250 rpm) overnight.

Colletotrichum 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 2. Organisms and Constructions Used in *In Vivo* Experiments

Phytopathogens	Plants	Constructions and Their Delivery into the Plants	
Bacteria			
Clavibacter michiganensis subsp. *sepedonicus* AS1	*Solanum tuberosum*	pP2C2S pP2C2S/*sap*	transcript infection
Pseudomonas syringae pv. *syringae* 61	n/a	n/a	n/a
Pseudomonas syringae pv. *tabaci* 11528 Race 0	n/a	n/a	n/a
Fungus			
Colletotrichum coccoides	*Nicotiana benthamiana*	pP2C2S pP2C2S/*sap* or	transcript infection
		pGR107 pGR107/*sap*	agroinfiltration

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 1×10^4 CFU per ml for *C. michiganensis*, 4×10^5 CFU per ml for *P. syringae* pv. *syringae* and 8×10^4 CFU per ml for *P. syringae* pv. *tabaci.*. The reaction mixtures were incubated at 28ºC 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^{-6}) 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 immersion in bacterial suspension (7×10^6 CFU/ml) for 30 min followed by planting in soil. Bacterial suspension (7 days old) was centrifuged at 20°C for 15 min at 4000 rpm; with which the cell pellet was washed twice with and finally resuspended in sterile water. The plants were grown in green house at 27±2°C under 14-h/10-h light/dark photoperiod.

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 28ºC 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 in Plant Tissues

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 IS*1121*, 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 in 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 (25ºC)

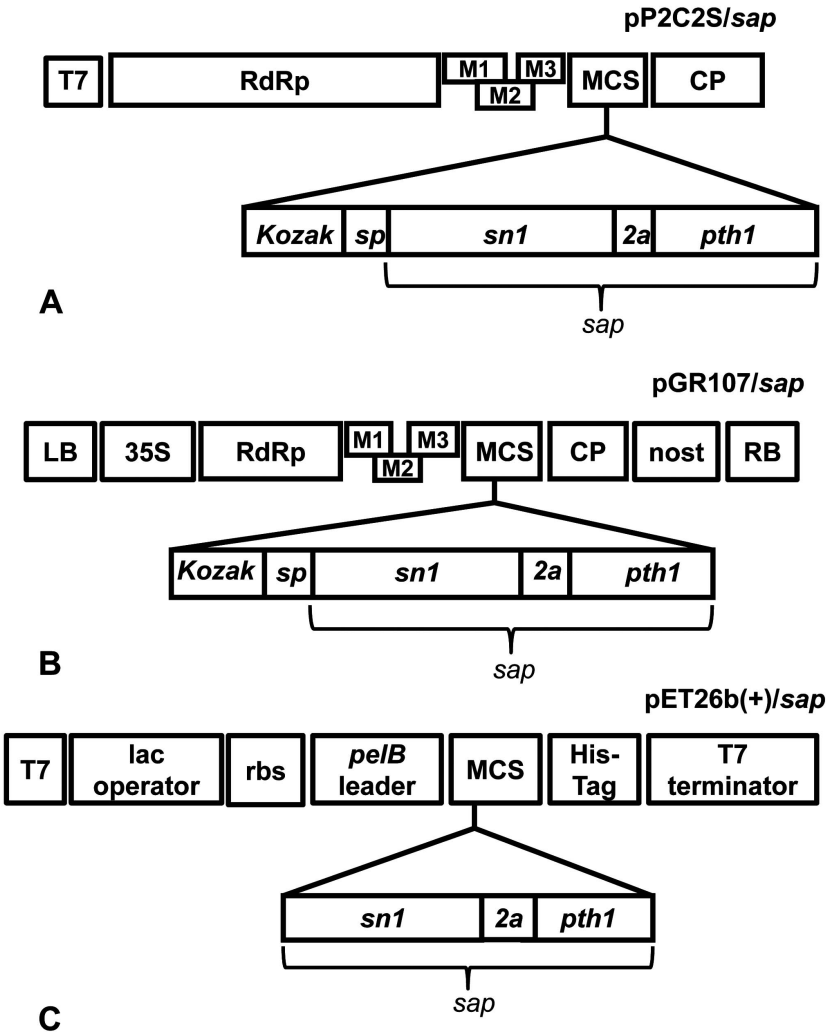

Fig. (1). Schematic representation of vector constructions carrying engineered genes *sn1* and *pth1* in the form of hybrid gene *sap.* The cloning/expression region of pET-26b(+)/*sap* vector indicating the insertion of the *sap* gene. (**A**). pET-26b(+) carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal His-Tag sequence. rbs – ribosomal binding site sequence. Genome organization of PVX-based vectors carrying the *sap* gene: pP2C2S/*sap* (**B**) and pGR107/*sap* (**C**). Features shown are: T7 bacteriophage (T7) and *Cauliflower mosaic virus* 35S (35S) promoters; viral RNA-dependent RNA polymerase gene (RdRp); "triple gene block" encoding three specific movement proteins of PVX: M1, M2, M3; multiple cloning site (MCS); coat protein (CP); nopaline synthase transcriptional terminator (*nos*t); T-DNA left and right borders (LB, RB); genes for signal peptide (*sp*) to facilitate the localization of hybrid to the periplasm, SN1 (*sn1*), FMDV 2A protease oligopeptide (*2a*), PTH1 (*pth1*) and SAP (*sap*).

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

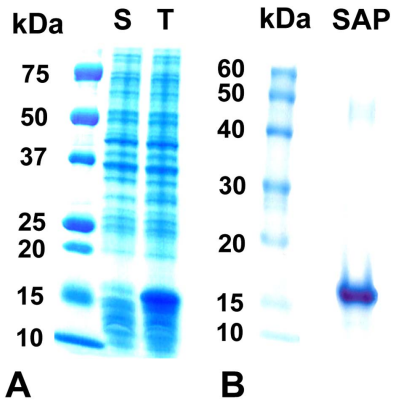

Fig. (2). Denaturing 10-20% polyacrylamide gel electrophoretic analysis of **A**: soluble (S) and total (T) fractions of SAP produced in *E. coli* transformed by pET26b(+)/*sap*. Sample lanes contain 4 µg (S) and 5 µg (T) of protein. kDa= Precision Plus Protein Kaleidoscope standards (Bio-Rad). The gel was stained with SimplyBlue Safe Stain (Invitrogen); **B**: SAP after IB purification, solubilization and protein refolding. Sample lane contains 10µg of SAP. kDa= BenchMark™ His-tagged Protein standards (Invitrogen).

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 pGR107/*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

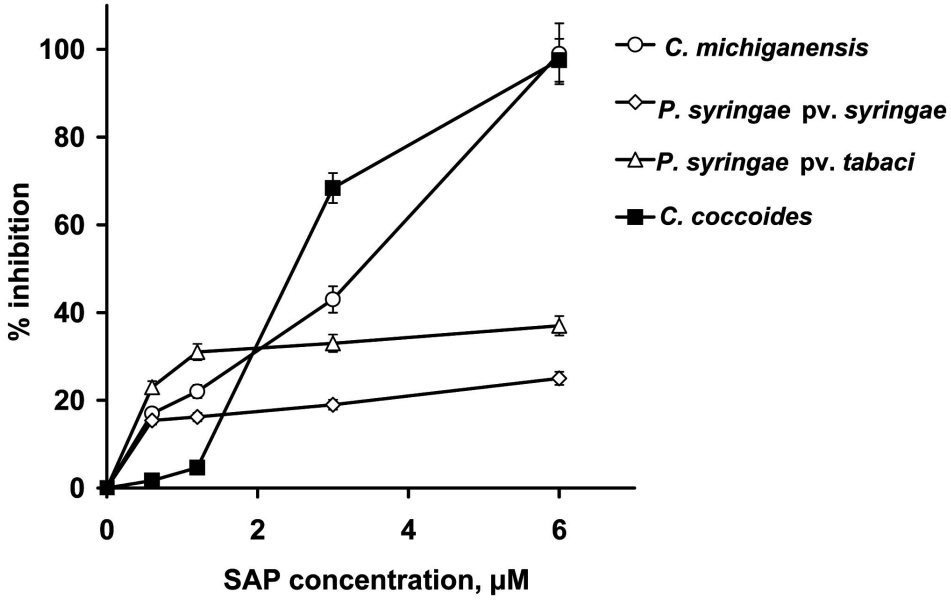

Fig. (3). The antimicrobial effect of SAP on various phytopathogenic microorganisms *in vitro*. % inhibition on the Y-axis refers to colony-forming units (CFU) for bacteria (*C. michiganensis*, *P. syringae* pv. *syringae, P. syringae* pv. *tabaci*) and spore germination for fungus *C. coccoides*. Each result is a mean of three replications. Values are expressed as means ± 5%.

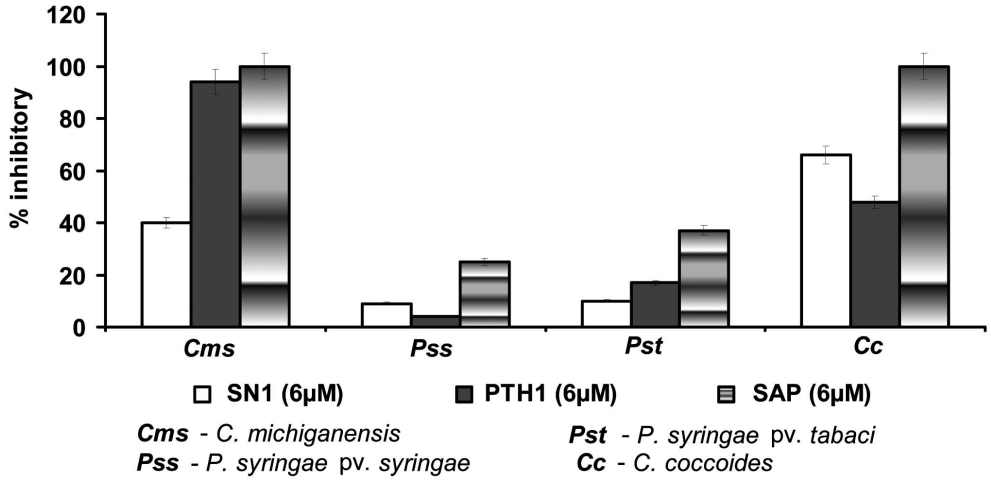

Fig. (4). Comparison of antimicrobial activities of individual SN1 and PTH1 with activity of hybrid SAP against phytopathogens. % inhibition on the Y-axis refers to CFU for bacteria (*C. michiganensis*, *P. syringae* pv. *syringae, P. syringae* pv. *tabaci*) and spore germination for fungus *C. coccoides*. Each result is a mean of three replications. Values are expressed as means ± 5%.

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.

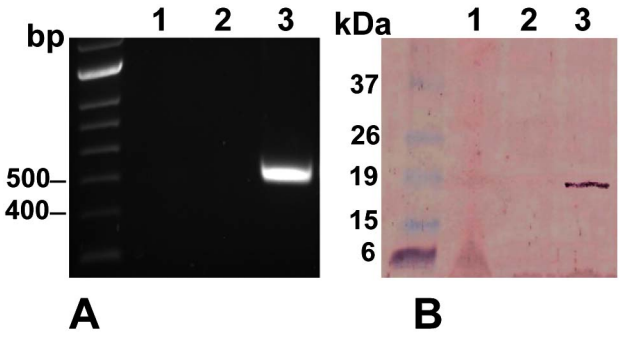

Fig. (5). Detection of the *sap* gene and its expression in agroinfiltrated tobacco plants. **A**: RT-PCR analysis of total cellular RNA extracted from systemically infected leaves (7th day after agro-infiltration). Lane 1: "control-mock"; lane 2:"empty-pGR107"; lane 3: "pGR107/*sap*". **B**: Western blot analysis of total protein fraction extracted from systemically infected leaves (49th day after agro-infiltration). Lane 1: "control-mock"; lane 2: "empty-pGR107"; lane 3: "pGR107/*sap*". The blot was incubated with anti-SN1PTH1 polyclonal antisera followed by alkaline phosphatase-conjugated anti-rabbit sera.

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 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 pRG107/*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*).

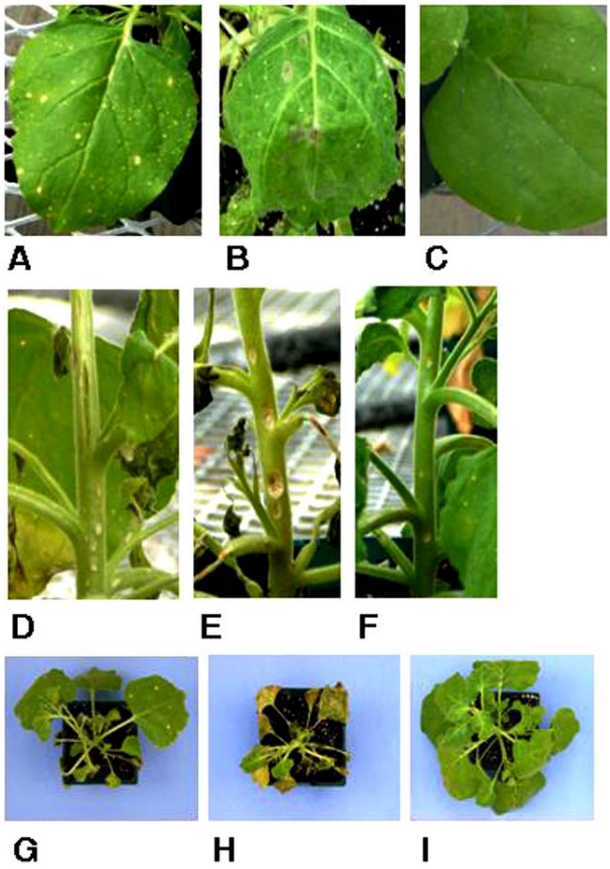

Fig. (6). Anthracnose symptoms on tobacco plants inoculated by *C. coccoides*. Fully expanded leaves of "control-mock" (**A**), "empty-pP2C2S" (**B**) and "pP2C2S/*sap*" (**C**) groups (9th day after fungal inoculation). The fragments of the tobacco stems of "control-mock" (**D**), "empty-pP2C2S" (**E**) 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).

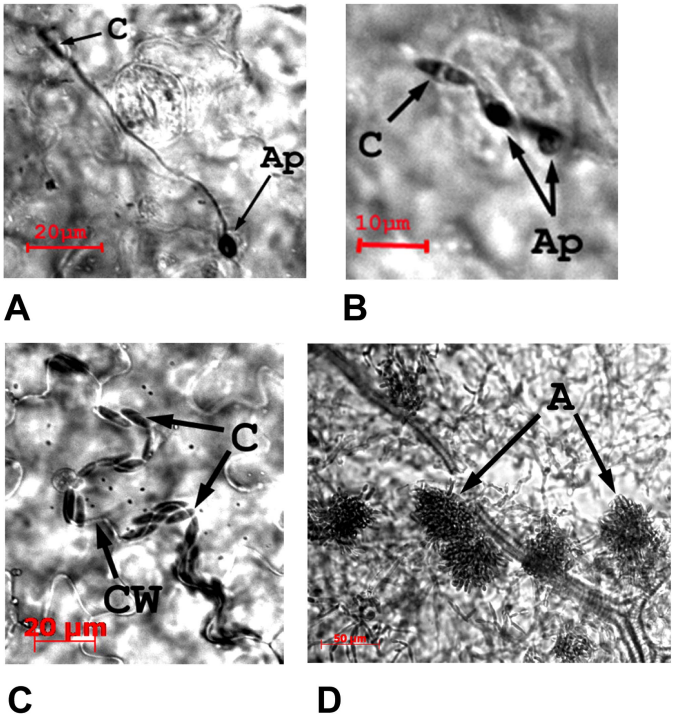

Fig. (7). Sections of the tobacco leaf surfaces stained with lactophenol cotton blue solution. Germination of conidia (C) of *C. coccoides* with appressorium (Ap) formation in "control-mock" (**A**) and "empty-pGR107" (**B**) groups (2nd dpi). **C**: specific localization of fungal conidia along the plant cell wall (CW) in "pP2C2S/*sap*" group (2nd dpi). **D**: Acervulus (A) development in "control-mock" group (5th dpi).

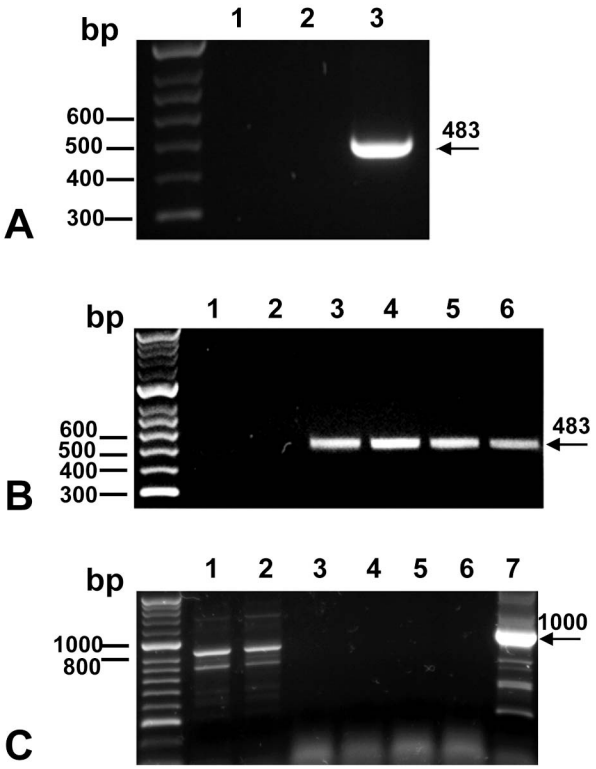

Fig. (8). Molecular analysis of potato plants. RT-PCR analysis using primers SN1F/PTH1R of RNA samples isolated from the systemically infected leaves (**A**) (14th day after rub-infection) and tubers (**B**) (56th day after rub-infection). **C**: PCR analysis of DNA samples isolated from the infected tubers with primer pairs specific to *C. michiganensis* subsp. *sepedonicus* genome (40th dpi by *C. michiganensis*). Lane 1: "control-mock", lane 2: "empty-pP2C2S", lanes 3-6: "pP2C2S/*sap*", lane 7: *C. michiganensis* (positive control). bp = AlphaQuant™2 (Alpha Innotech). Arrows indicate the size of the expected PCR amplicon in base pairs.

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 micro-biological 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 prokaryotes 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

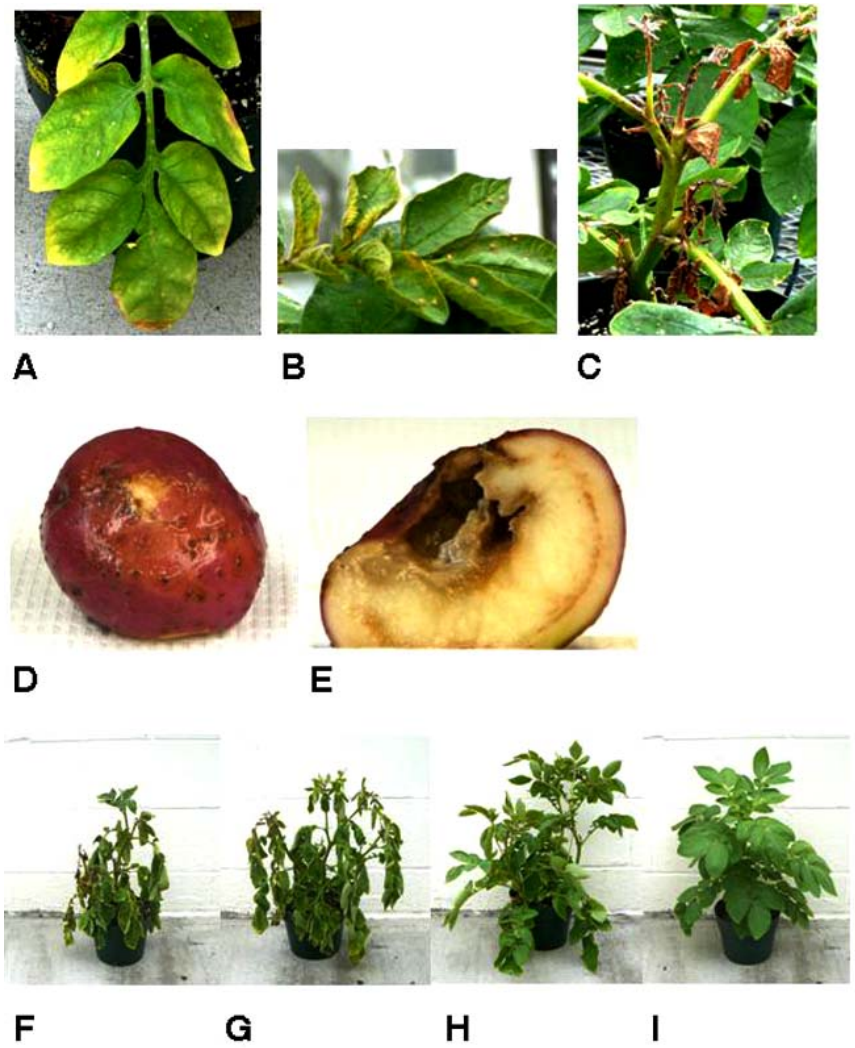

Fig. (9). Foliar and tuber symptoms of potato ring rot disease, caused by *C. michiganensis.* Above-ground symptoms of potato ring rot disease on plants of "control-mock" (**A-B**) and "empty-pP2C2S" groups (**C**) (37/28 dpi, respectively; the plants were infected by root trimming). Surface (**D**) 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

sap gene in tobacco plants was confirmed for *Agrobacterium*-mediated gene transfer. In this case, we employed the advantage of *Agrobacterium* system [63] and co-expressed in tobacco plants *sap* gene and *p19* gene encoding the p19 protein of *Tomato bushy stunt virus* (TBSV), a powerful silencing suppressor protein [64-66], to boost production of the SAP protein.

Although the appearance of anthracnose symptoms on tobacco plants, caused by the fungus *C. coccoides*, was observed in all experimental groups, the severity was significantly lower in plants expressing *sap* gene (Fig. **6**). These data strongly suggest a plant protective role of SAP protein against fungal infection.

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-pP2C2S" 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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