

Snakin-2, an Antimicrobial Peptide from Potato Whose Gene Is Locally Induced by Wounding and Responds to Pathogen Infection¹

Marta Berrocal-Lobo, Ana Segura², Manuel Moreno, Gemma López, Francisco García-Olmedo, and Antonio Molina*

Laboratorio de Bioquímica y Biología Molecular, Departamento de Biotecnología-Universidad Politécnica Madrid, Escuela Técnica Superior Ingenieros Agrónomos, E-28040 Madrid, Spain

The peptide snakin-2 (StSN2) has been isolated from potato (*Solanum tuberosum* cv Jaerla) tubers and found to be active ($EC_{50} = 1-20 \mu M$) against fungal and bacterial plant pathogens. It causes a rapid aggregation of both Gram-positive and Gram-negative bacteria. The corresponding *StSN2* cDNA encodes a signal sequence followed by a 15-residue acidic sequence that precedes the mature StSN2 peptide, which is basic (isoelectric point = 9.16) and 66 amino acid residues long (molecular weight of 7,025). The *StSN2* gene is developmentally expressed in tubers, stems, flowers, shoot apex, and leaves, but not in roots, or stolons, and is locally up-regulated by wounding and by abscisic acid treatment. Expression of this gene is also up-regulated after infection of potato tubers with the compatible fungus *Botrytis cinerea* and down-regulated by the virulent bacteria *Ralstonia solanacearum* and *Erwinia chrysanthemi*. These observations are congruent with the hypothesis that the StSN2 is a component of both constitutive and inducible defense barriers.

An important component of plant defense is a diverse set of constitutive and pathogen-inducible antimicrobial compounds that includes the so-called pathogenesis-related proteins, several families of antimicrobial peptides, a variety of chemically diverse organic compounds classified as phytoalexins and phytoanticipins, and certain active oxygen and nitrogen species (Osbourn, 1996, 1999; Broekaert et al., 1997; Kombrink and Somssich, 1997; García-Olmedo et al., 1998, 2001). Accumulation of these compounds and the ability of a given pathogen to deal with them may be decisive for the outcome of the interaction (Titarenko et al., 1997a; López-Solanilla et al., 1998, 2001; Miguel et al., 2000; Alamillo and García-Olmedo, 2001; García-Olmedo et al., 2001). Thus, it has been shown that increased levels of certain antimicrobial peptides, either through overexpression of the corresponding genes or by appropriate exogenous treatments, result in enhanced tolerance to particular pathogens (Carmona et al., 1993; Terras et al., 1995; Epple et al., 1997; Molina and García-Olmedo, 1997; Holtorf et al., 1998; Thomma et al., 1998, 1999).

Furthermore, pathogen mutants that are sensitive to antimicrobial peptides show decreased virulence when inoculated in the plant (Titarenko et al., 1997a; López-Solanilla et al., 1998).

Several families of antimicrobial peptides have been characterized in plants (García-Olmedo et al., 1992, 1995, 1998, 2001; Broekaert et al., 1997). The majority of them are Cys-rich and their globular structure is stabilized by disulphide bridges, although linear Gly-/His-rich and macrocyclic Cys-knot peptides have also been recently identified (Tam et al., 1999; Park et al., 2000). The peptides are generally encoded by multigenic families in which some genes are developmentally regulated in certain tissues, whereas others are pathogen inducible, and a number of them show both constitutive and pathogen-inducible expression (García-Olmedo et al., 1995, 1998, 2001; Broekaert et al., 1997).

In a previous report, a novel 12-Cys, antimicrobial peptide from potato (*Solanum tuberosum* cv Jaerla) tubers, snakin-1 (StSN1), was described (Segura et al., 1999). Its amino acid sequence was homologous to those deduced from a number of anonymous cDNAs reported in different plant species (Shi et al., 1992; Herzog et al., 1995; Ben-Nissan and Weiss, 1996; Kotilainen et al., 1999). Most of the corresponding genes are constitutively expressed, and some of them (*GAST1* from tomato [*Lycopersicon esculentum*; GA-stimulated transcript] and *GASA1* and *GASA4* from Arabidopsis [GA stimulated in Arabidopsis]) have been shown to be up-regulated by gibberellic acid (GA) in GA-deficient mutants and, to a lesser degree, in wild-type plants (Shi et al., 1992; Herzog et al.,

1995; Ben-Nissan and Weiss, 1996; Kotilainen et al., 1999).

The *StSN1* gene from potato is constitutively expressed in different tissues during development and does not respond to GA and other abiotic or biotic treatments (Segura et al., 1999). Here, we report a second snakin peptide (StSN2) from potato that represents a quite divergent (38% conserved residues) snakin/GASA subfamily. Its spectrum of antimicrobial activity against the bacterial and fungal pathogens tested is quite similar to that of StSN1 and different from that of defensin peptides from the same tissues. However, expression of the *StSN2* gene is locally induced by wounding and shows differential responses to pathogen infection, which is in contrast with that of the *StSN1* gene. Expression patterns and antimicrobial activities of StSN2 are congruent with its possible participation in both constitutive and inducible defense barriers of potato.

RESULTS

Isolation and Characterization of StSN2

An HPLC fraction with antimicrobial activity (StSN2; Fig. 1A) was isolated from a crude cell wall extract that was obtained from potato tubers as previously described (Moreno et al., 1994; Segura et al., 1999). This fraction was homogeneous, as judged by SDS-PAGE, and comigrated with snakin-1 (StSN1; Segura et al., 1999; Fig. 1B). The homogeneity of this fraction was confirmed by matrix-assisted laser desorption/ionization (MALDI)-time of flight mass spectrometry (MS), which detected a unique compound with a molecular mass of 7,024.93 D. The peptide was named snakin-2 (StSN2) because its N-terminal amino acid sequence, determined up to the 17th residue by Edman degradation, indicated homology to snakin-1, as well as to deduced amino acid sequences of the GASA family. The concentration of StSN2 in tubers was estimated to be in a range of 2 to 4 $\mu\text{mol kg}^{-1}$ fresh weight.

To clone the *StSN2* cDNA, 3'/-5'-RACE was carried out, using tuber cDNA as template. The full-length cDNA of *StSN2* encoded a protein with a signal peptide sequence (residues 1–23), followed by a 15-residue-long acidic peptide ($\text{pI} = 3.1$) and then by a 66-residue sequence whose N-terminal was identical to that directly determined by Edman degradation of the purified peptide (Fig. 1C). The mature peptide was basic ($\text{pI} = 9.6$) and its calculated molecular mass was 7,025.14 D (assuming that the 12 Cys were in disulphide form), a figure that differs by less than 1 D from that directly determined by MALDI-time of flight MS.

Amino acid sequence alignments shown in Figure 2A indicate that StSN2 represents one of the three subfamilies into which snakin/GASA sequences can

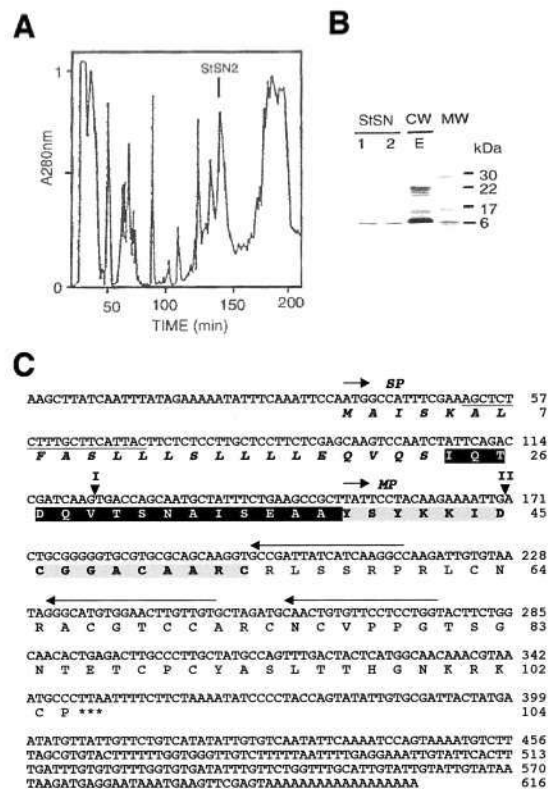


Figure 1. Purification and characterization of StSN2. A, reverse phase-HPLC fractionation of the cell wall extract (CWE) from potato tubers. The linear gradient used was water (0.1% [v/v] trifluoroacetic acid)-2-propanol, 0% to 30% for 180 min and 30% to 50% for 15 min. Fraction corresponding to StSN2 is indicated. B, Separation by SDS-PAGE of the purified proteins StSN2 and StSN1 (Segura et al., 1999), and CWE from potato tuber. Molecular mass markers (MW) are indicated. C, Nucleotide sequence of *StSN2* cDNA (AJ312904) and amino acid sequence of the corresponding protein. The gray-shaded amino acid sequence was obtained by direct N-terminal Edman degradation of the purified StSN2 and the rest of protein sequence was deduced from the cDNA sequence. Signal peptide (SP) is followed by a black-shaded amino acid sequence corresponding to the acidic sequence that preceded StSN2 mature peptide (MP). Predictions of SP were done by using the SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) and Psort (<http://psort.nibb.ac.jp/>) program. Oligonucleotide sequences used for 5'-RACE are indicated by horizontal arrows, and those used for PCR amplification of the *StSN2* gene are underlined. The position of the introns (I and II) in the *StSN2* gene (AJ312424) are indicated by triangles.

be classified. In addition to the 12 characteristic Cys, residues at nine positions are highly conserved throughout the family, whereas a number of motifs define each of the three subfamilies (Fig. 2A). StSN2 also shows some sequence similarity with Cys-rich domains from animal proteins, such as von Willebrand factors (vWF; Shelton-Inloes et al., 1986; Verweij et al., 1986), mucins (Li et al., 1998), and MDC (metalloproteinase-like desintegrin-like Cys-rich) proteins (Wolfsberg et al., 1995; Sagane et al., 1998; Fig. 2B). Although plant snakin peptides share certain motifs with disintegrin hemotoxic snake venoms, they lack

A

	1	10	20	30	40	50	60								
StSN2	YSYKKID	CGGACAAR	CRLSSRPRL	CNRAC	GTCCAR	CNCVPE	PGTSGNTE	TCPYASLTTHGNKRKCP							
LsGASAI1	DSYKKID	CGGACAAR	CRLSSRPRL	CHRA	CGTCCAR	CNCVPE	PGTSGNTE	TCPYASLTTHGNKRKCP							
AtGASA1	GYAKKID	CGSACVAR	CRLSSRPRL	CHRA	CGTCCYR	CNCVPE	PGTYGNYDK	CCYASLTTHGGRRKCP							
FaGAST	SLLAKID	CGGACKAR	CRLSSRPRL	CKRAC	GTCCQR	CSCVPE	PGTAGNYDV	CPYATLTTHGGRRKCP							
GhGEG	IAASKIN	CGAACKAR	CRLSSRPRL	CHRA	GTCCAR	CRCVPE	PGTSGNQK	VCPYINMTTHGGRRKCP							
StSN1	GSNFC	DSKCKL	RCSKAGLAD	RCLKY	CGIC	CEEECK	CVPSGTY	GNKHECPYRDKKNSKGSKCP							
LsGASAI	GSYFC	DSKCKL	RCSKAGLAD	KLYCE	IC	CEEECK	CVPSGTY	GNKHECPYRDKKNSKGSKCP							
Rc153	KSLFC	ANKCND	RCARAGV	KDRCKI	KYCE	IC	CAECK	CVPSGTYGNKHECPYRDKKNSKGSKCP							
AtGASA7	LEKWC	GGQKCE	GRCKEAG	MKDRCL	KYCG	IC	CKDC	CVPSGTYGNKHECACYRDKLSKGTTPKCP							
AtGASA8	DS	CGGKCN	VRC	SKAGQ	HEECL	KYCN	IC	CKKCN	CVPSGTFGNKDECPYRDMKNSKGGSKCP						
LsGAST1	GRLHPQD	CQPKCT	YRCSKTS	SFKKPC	MF	FCQ	CCAKCL	CVPEACTYGNKQTCPCYNNWTKRGGPKCP							
StSNIII	YNKLRPRD	CKPKCT	YRCSAT	SHKKPC	MF	FCQ	CCATCL	CVPEKGVYGNKQSCPCYNNWTKQEGPKCP							
PhGIP1	GSLHPQD	CQPKCT	YRCSKTS	SFKKPC	MF	FCQ	CCAKCL	CVPEAGTYGNKQTCPCYNNWTKRGGPKCP							
AtGASA4	GSLKRTQ	CPSECD	RRCKKT	QYHKAC	IT	FC	NKCCR	KCLCVPEGGYGNKQVSCPCYNNWTKQEGGPKCP							
PmGAST1	GSLRPS	ECGQRC	SYRCSAT	SMKKPC	H	FF	CCAKCL	CVPEGTFGNKQVCPYNNWTKQGGPKCP							
Consensus		C	C	RC		C	C	CC	C	CVP	G	GN	C	CY	KCP

B

StSN2 (7-49)	CGGACAAR	CRLSSRPRL	CNRAC	GTCCA	RCNCV	PEPGT	SGNTE	TCP
HsvWF (660-721)	CGTFCN	LTCSR	LSY	PEDEEC	N	EAC	LE	GC
BtvWF (303-341)	CVSP	CHRT	CRSL	SIT	EV	CRE	Q	VD
HsMuc (340-378)	CGSP	CVDT	CSNP	QHSQV	CE	DH	CV	AG
StSN2 (11-66)	CAA	RCRL	SSRPRL	CNRAC	GTCCAR	CNCVPE	PGTSGNTE	TCPYASLTTHGNKRKCP
HsMDC (473-524)	CKK	KCTL	THDAM	C	SD	GL	CCRR	CKY
HsMDC2a (473-524)	CKK	KCTL	TQDSQ	C	SD	GL	CC	KK
GbDIS (16-67)	CD	AAT	CKLR	QGAQ	C	AE	GL	CC

Figure 2. Alignment of snakins/GASAs amino acid sequences. A, Comparison of amino acid sequences of snakins/GASAs of subfamilies II, I, and III, respectively. Amino acids conserved across all the family members are black shaded and indicated in the consensus. Highly conserved residues that are relevant for subfamily classification (conserved in known, non-represented members of each group) are gray shaded. Sequences taken for the alignment are the following: StSN1 (Segura et al., 1999) and StSNIII (BG597515) from potato; AtGASA1 and AtGASA4 (Herzog et al., 1995), AtGASA7 (AC005396.2), and AtGASA8 (AC004218.2) from Arabidopsis; LsGAST1 (Shi et al., 1992), LsGASAI (BG130738), and LsGASAI1 (A177478) from tomato; GhGEG from *Gerbera hybrida* (Kotilainen et al., 1999); FaGAST from *Fragaria ananassa* (AF039183); Rc153 from *Ricinus communis* (EMBL T24153); PhGIP1 from *Petunia hybrida* (Ben-Nissan and Weiss, 1996); and PmGAST1 from *Picea marina* (AF051227). The putative N-terminal sequences of the proteins have been determined by homology with those of StSN1 and StSN2, and by using the posttranslational predictions programs indicated in Figure 1C. B, Alignment of potato StSN2 sequence with amino acid sequence of Cys-rich domains of proteins from mammals and the hemotoxic snake venom disintegrin. Identical residues are black shaded and similar, conservative positions (polar, apolar, and charged) are gray shaded. Proteins used in the alignment are the following: HsvWF (X04385), HsMuc (Q9ESP3), HsMDC, and HsMDC2a (Sagane et al., 1998) from human; BtvWF from *Bos taurus* (P80012); and disintegrin from agkistrodon halys blomhoffii snake (*Gloydius blomhoffii*; P21858). A stretch of 15 amino acids of HsvWF that has not been included in the alignment is indicated by an asterisk. The RGD motif of disintegrin essential for protein action is underlined. Similarities between StSN2 and the aligned proteins were found using the Jpred program for secondary structure predictions (<http://barton.ebi.ac.uk/>).

the RGD motif that is responsible for disintegrin activity (Fig. 2B; Dennis et al., 1990).

Activity against Bacterial and Fungal Pathogens

In Table I, the in vitro antimicrobial activity of purified StSN2 can be compared with that of StSN1 and defensin StPTH1, which also accumulate in potato tubers (Moreno et al., 1994; Segura et al., 1999). StSN2 was active ($EC_{50} = 1-20 \mu\text{M}$) against all the fungal species tested, the Gram-positive bacterium *C.*

michiganensis subsp. *sepedonicus*, and the Gram-negative bacterium *R. meliloti*, whereas it was inactive against the Gram-negative bacteria *R. solanacearum* and *E. chrysanthemi* (Table I). Despite their divergent amino acid sequences (38% identical residues), StSN2 and StSN1 antimicrobial activity spectra were essentially equivalent (Table I). In contrast, there were significant differences with respect to that of defensin StPTH1. For example, *F. culmorum* and *R. meliloti* were resistant to StPTH1, but sensitive to StSN2, and *F. solani* was 15 times more sensitive to

Table 1. Inhibition of bacterial and fungal pathogens by snakins *StSN2* and *StSN1* and defensin *StPTH1* from potato

Pathogen	Protein (EC ₅₀) ^a		
	<i>StSN2</i>	<i>StSN1</i>	<i>StPTH1</i>
	μM		
Bacteria			
<i>Clavibacter michiganensis</i>	1	4	7
<i>Ralstonia solanacearum</i>	NA	NA	25
<i>R. solanacearum</i> (<i>rfa</i> ⁻)	30	15	25
<i>Erwinia chrysanthemi</i>	NA	NA	NA
<i>Rhizobium meliloti</i>	8	nt	NA
Fungi			
<i>Botrytis cinerea</i>	2	0.8	1
<i>Fusarium solani</i>	3	2	0.2
<i>Fusarium culmorum</i>	2	2	NA
<i>Fusarium oxysporum</i> f. sp. <i>conglutinans</i>	10	13	nt
<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	20	13	nt
<i>Plectosphaerella cucumerina</i>	10	10	10
<i>Colletotrichum graminicola</i>	10	20	2
<i>Colletotrichum lagenarium</i>	10	10	nt
<i>Bipolaris maydis</i>	20	20	10
<i>Aspergillus flavus</i>	20	20	nt

^a EC₅₀, Effective concentration for 50% inhibition; NA, not active at concentration <20 μM ; nt, not tested.

StPTH1 than to *StSN2* (Table I). Similar to *StSN1* and in contrast with other types of antibiotic peptides, *StSN2* caused rapid aggregation of both Gram-positive and Gram-negative bacteria (Fig. 3), although this property did not correlate with its inhibitory activity. Finally, as described for other plant antimicrobial peptides (De Samblanx et al., 1997; Se-

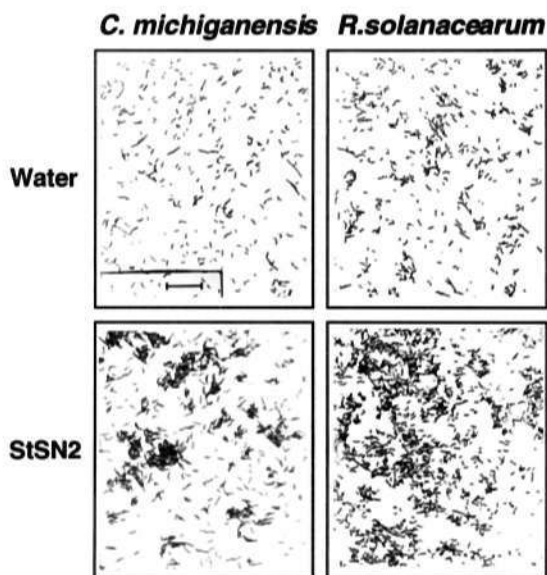


Figure 3. Aggregation of bacteria caused by potato *StSN2*. A 5- μL suspension (10^5 colony forming units [cfu] mL^{-1}) of the Gram-positive bacterium *C. michiganensis* subsp. *sepedonicus* or the Gram-negative bacterium *R. solanacearum* was deposited in a microscope slide, then 5 μL of a 20 μM solution of *StSN2* or a 5- μL drop of water was added, and a photograph was taken immediately under a microscope. Bar represents 20 μm .

gura et al., 1998, 1999), the in vitro activity of *StSN2* was reverted when salt (50 mM KCl + 1 mM CaCl_2) was added to the growth media (data not shown).

Developmental Expression of the *StSN2* Gene

The *StSN2* cDNA, which did not give any cross-hybridization with potato *StSN1* DNA (data not shown), was used for Southern-blot hybridization of potato DNA and the observed patterns were compatible with the presence of one or two copies of the gene in the genome (Fig. 4). PCR amplification and sequencing of *StSN2* demonstrated the presence of two introns that were 249 and 163 bp in length, respectively, and were in similar positions to those of other genes of the same subfamily (Fig. 1C).

The expression of *StSN2* gene in potato plants was analyzed and compared with that of the *StSN1* by the northern-blot technique. Steady-state levels of *StSN2* mRNA were high in tubers, as well as in petals, stamens, and carpels from fully developed flowers (Fig. 5). In all these organs except petals, *StSN1* was not highly expressed (Fig. 5). Conversely, low levels of *StSN2* mRNA were detected in stem, shoot apex, and flower buds where *StSN1* was highly expressed (Fig. 5). *StSN2*, but not *StSN1*, was expressed in leaves, where it reached a peak at emergence in 4-week-old plants and decreased at the start of senescence (Fig. 5; data not shown). *StSN2* mRNA was not detected in other tissues and organs analyzed, including roots, stolons, and sepals (Fig. 5).

Response of the *StSN2* Gene to Abiotic Treatments

Excised potato plants were treated with GA (50 μM) and abscisic acid (ABA; 100 μM) by stem feeding. As shown in Figure 6, mock treatment slightly increased the basal *StSN2* mRNA level. This effect was significantly enhanced in leaves and stems by ABA

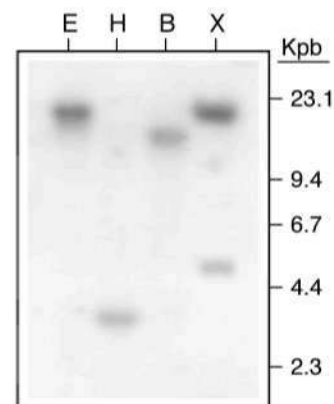


Figure 4. Southern-blot analysis of *StSN2* gene. Potato genomic DNA (10 μg) was digested with the *EcoRI* (E), *HindIII* (H), *BamHI* (B), or *XhoI* (X) restriction endonucleases. The *StSN2* cDNA fragment obtained by 5'-RACE (nucleotides 170–616) was [³²P] labeled and used as probe.

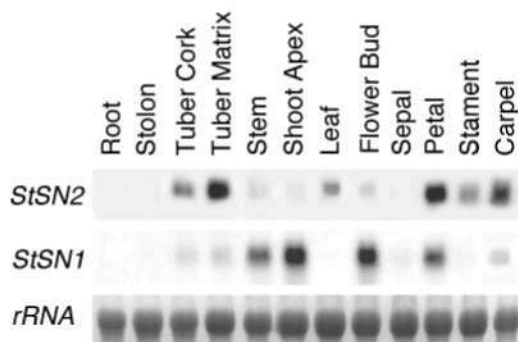


Figure 5. Expression of *StSN2* gene in potato. Northern-blot analysis of total RNAs (5 μ g) extracted from the indicated parts of the potato plant. Blot was hybridized with the *StSN2* probe and an *StSN1* probe (Segura et al., 1999). Equal loading was confirmed by rehybridization of the blot with a potato 18S-ribosomal RNA probe.

and prevented by GA (Fig. 6). Expression of the defensin *StPTH1* gene from potato (Moreno et al., 1994), used as a negative control, was also slightly increased in the mock experiment, but unaffected by either ABA or GA (Fig. 6).

Because it is known that some defensin genes are wound inducible (Broekaert et al., 1997), the concomitant induction of the *StSN2* gene in excised plants suggested that this gene could respond to mechanical damage. The enhanced expression of *StSN2* gene after treatment with ABA, which plays a role in wound signaling in solanaceas (Peña-Cortés et al., 1989; León et al., 2001), was also in line with a putative regulation of *StSN2* gene by wounding. To directly test this hypothesis, the experiment summarized in Figure 7 was done, using the wound-

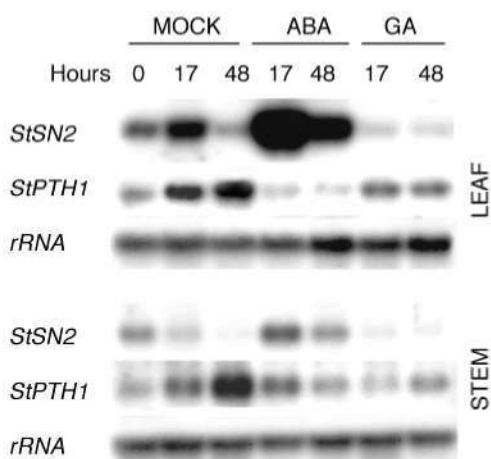


Figure 6. Expression of *StSN2* gene is induced by ABA. Northern-blot analysis of total RNAs (7.5 μ g) extracted from leaves and stems of mock-treated plants (M), or plants treated with 100 μ M ABA or 50 μ M GA₃. Blot was hybridized with the *StSN2* probe, and a potato defensin *StPTH1* probe (Moreno et al., 1994). RNA equal loading was confirmed by rehybridization of the blot with a potato 18S-ribosomal RNA probe. This is one of three experiments carried out that gave similar results.

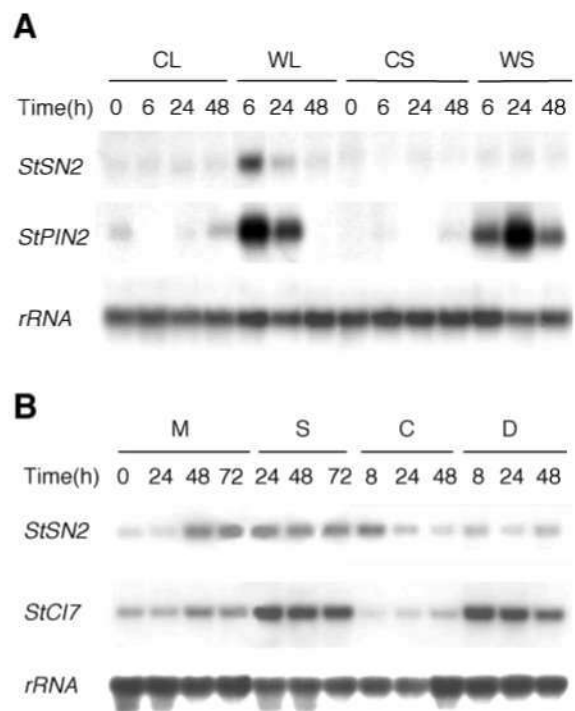


Figure 7. Response of *StSN2* gene to wounding and water-stress. A, Northern blot (7.5 μ g per sample) of total RNAs extracted from mechanically damaged leaves (WL) and upper systemic non-damaged leaves (WS) from wounded plants, and from same-age leaves of control, non-wounded plants (CL and CS). Blot was hybridized with the *StSN2* probe and with a potato *StPIN2* probe (Peña-Cortés et al., 1989). B, Northern-blot analysis of total RNAs (7.5 μ g) extracted from leaves of excised plants mock treated (M) or incubated with salt (250 mM NaCl; S), intact control plants (C), and plants that were remove from soil and were left to dry (drought treatment; D). Blot was hybridized with the *StSN2* probe, and a probe of the potato *Cl7* gene (Kirch et al., 1997). RNA equal loading was confirmed by rehybridization of the blot with a potato 18S-ribosomal RNA probe. This is one representative experiment of the two carried out that gave similar results.

inducible *StPIN2* gene as positive control (Peña-Cortés et al., 1989). Expression of the *StSN2* was locally induced in the wounded leaves, but not in the upper systemic, non-damaged leaves (Fig. 7A). This induction was not observed in mechanically damaged tubers and stems of potato (data not shown). Chitosan and jasmonic acid (JA), which have also been implicated in wound response (Bishop et al., 1981; Farmer and Ryan, 1990), had no effect on the expression of *StSN2* gene (data not shown).

Because it is known that ABA mediates adaptive responses to water stress (salinity or drought; Grill and Himmelbach, 1998), possible responses of the *StSN2* gene to this type of stress were investigated, using as control the *Cl7* gene, which responds to salinity and drought (Kirch et al., 1997). Gene *StSN2* was found to respond only weakly to the salinity treatment and was unaffected by drought (Fig. 7B).

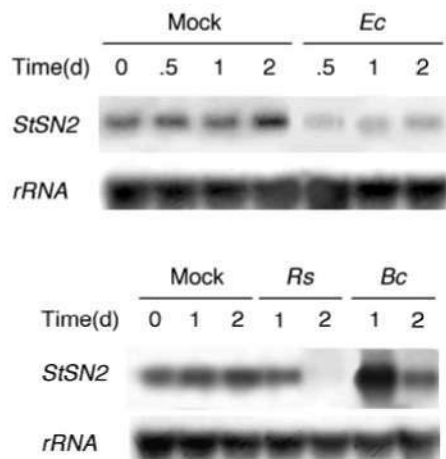


Figure 8. Response of *StSN2* gene to infection of potato tubers with pathogens. Northern blot (7.5 μg per sample) of total RNAs extracted from tubers mock inoculated with 10 mM MgCl_2 (M), infected with bacterial suspensions (50 μL of 10^7 cfu mL^{-1}) of *R. solanacearum* (*Rs*) or *E. chrysanthemi* (*Ec*), or inoculated with a spore suspension (50 μL of 2×10^5 spores mL^{-1}) of the fungus *B. cinerea* (*Bc*). Blot was hybridized with the *StSN2* probe and RNA equal loading was confirmed by rehybridization of the blot with a potato 18S-ribosomal RNA probe. This is one of two experiments carried out that gave similar results.

Responses of the *StSN2* Gene to Infection by Bacterial and Fungal Pathogens

To investigate possible effects of pathogen infection on the expression of the *StSN2* gene, potato tubers were inoculated with the bacterial pathogens *R. solanacearum*, which induces tuber necrosis 1 d after inoculation (Titarenko et al., 1997a), and *E. chrysanthemi*, which causes soft rot disease in potato and a wide range of plants (Boccardo et al., 1991; López-Solanilla et al., 1998), or the fungus *B. cinerea*, which causes the gray mold disease in potato (Agrios, 1997). The gene was down-regulated by the two bacterial infections, whereas it was transiently induced after inoculation with the fungus (Fig. 8). In contrast, expression of the gene was unaffected when leaves were infected with the fungus or treated with ethylene (125 $\mu\text{L L}^{-1}$), a hormone involved in activation of some defense responses (Solano and Ecker, 1998; Thomma et al., 1998, 1999), or with benzo[1,2,3]-thiadiazole-7-carbothioic acid *S*-methyl ester (0.3 mM), a compound that induces systemic acquired resistance (Ryals et al., 1996; data not shown).

DISCUSSION

We have previously characterized the antimicrobial properties of the cell wall associated peptide snakin-1 (*StSN1*) from potato, the first purified member of what appears to be a widely distributed peptide type, the snakin/GASA family (Segura et al., 1999). This peptide was developmentally accumulated in different tissues of potato plants and the

expression of its corresponding gene was unaffected by a variety of abiotic or biotic challenges (Segura et al., 1999). We have now described the isolation of a new peptide from potato (*StSN2*) that represents a widely divergent snakin subfamily. The *StSN2* peptide is also active in vitro against bacterial and fungal plant pathogens and, in contrast with *StSN1*, expression of the corresponding gene is affected by certain external treatments including pathogen infection.

Like the majority of globular antimicrobial peptides from plants described so far (Broekaert et al., 1997; García-Olmedo et al., 1998, 2001), the snakin/GASA peptides are basic and rich in Cys residues, which may form six disulphide bridges that stabilize their structure. The amino acid sequences deduced from cloned snakin/GASA cDNAs and genes can be classified into three subfamilies (I–III). *StSN1* and *StSN2* are respectively the only purified members representing subfamilies I and II, whereas no member of subfamily III has been isolated so far.

Although *StSN1* and *StSN2* show only 38% sequence similarity, they have almost identical antimicrobial activity spectra. The concentration of *StSN2* in tubers and in other tissues and organs of the potato plant seems to be well above that possibly required for in planta inhibition of some pathogens, based on in vitro inhibitory activity of this peptide. In potato tubers, other antimicrobial peptides, such as defensin *StPTH1* (Moreno et al., 1994) and snakin *StSN1* (Segura et al., 1999), accumulate at high concentrations together with *StSN2*. The complementarity of the antibiotic activity spectra of defensin *StPTH1* and the snakins, as well as the synergism between the two peptide types (Moreno et al., 1994; Segura et al., 1999; M. Berrocal-Lobo, G. López, F. García-Olmedo, and A. Molina, unpublished data), suggests that the concomitant accumulation of snakins and defensins in a given tissue (i.e. in tubers or in flowers) may represent a constitutive, broad-spectrum, effective barrier against bacterial and fungal pathogens.

StSN2 causes a rapid aggregation of Gram-positive and Gram-negative bacteria in vitro, as previously reported for *StSN1* (Segura et al., 1999). The aggregation did not correlate with antimicrobial activity, but could play a role in the control of the pathogen in vivo because snakin peptides show sequence similarity with Cys-rich domains of proteins from animals that are involved in protein-protein interactions, such as the vWFs (Shelton-Inloes et al., 1986; Verweij et al., 1986), mucins (Li et al., 1998), and MDC proteins (Wolfsberg et al., 1995; Sagane et al., 1998). In particular, mucins coat the epithelia of intestines, airways, and other mucus membrane-containing organs from animals, providing a protective, lubricating barrier against particles and infectious agents (Pérez-Vilar and Hill, 1999). The mechanism of action of snakins remains unknown, but, in contrast with other antibiotic peptides from plants, they do not

interact with artificial lipid membranes (Caaveiro et al., 1997).

The *StSN2* mRNA encodes a precursor with a signal peptide, which is congruent with the cell wall location of the mature peptide, followed by an N-terminal acidic pro-peptide, 15 amino acid residues in length. Acidic peptides at the N terminus can be also predicted for other members of the family (e.g. AtGASA1, AtGASA4, GhGEG, and LsGAST1), but not for *StSN1*. It has been suggested that the acidic amino acid sequence that sometimes precedes or follows different antibiotic peptides may act as an inhibitor of endogenous toxicity during transport (see García-Olmedo et al., 2001).

Though constitutively expressed, the *StSN2* gene is up-regulated by ABA and down-regulated by GA, just the opposite of what has been described for other GASA genes, such as *GASA1* from Arabidopsis and *GAST1* gene from tomato, which are up-regulated by GA, and down-regulated by ABA (Shi et al., 1992; Herzog et al., 1995; Raventos et al., 2000). GA has been shown to down- or up-regulate the expression of the Arabidopsis *GASA4* gene in a tissue-specific manner (Aubert et al., 1998). ABA is known to mediate some plant responses to environmental stress, including osmotic stress produced by cold, high salinity, or drought, as well as to mechanical injury produced by wound-causing agents (Peña-Cortés et al., 1989, 1995; Grill and Himmelbach, 1998; León et al., 2001). However, the *StSN2* gene is not a typical water stress-inducible gene such as tomato *TAS14* (Godoy et al., 1990) or potato *CI7* (Kirch et al., 1997) because it did not respond clearly to salinity or drought.

StSN2 expression was locally induced in wounded leaves, but not in systemic non-damaged leaves, and it did not respond to JA treatment. Both JA-dependent and -independent wound signal transduction pathways have been described in plants (Farmer and Ryan, 1990; Peña-Cortés et al., 1995; O'Donnell et al., 1996; Dammann et al., 1997; Reymond et al., 2000; León et al., 2001), and wound-inducible genes that do not respond to JA have been identified (e.g. *WR3* and *Twi1*; Nishiuchi et al., 1997; Titarenko et al., 1997b; O'Donnell et al., 1998; Reymond et al., 2000). The *StSN2* gene belongs to the latter class, but its expression differs from that of tomato *Twi1*, which shows systemic activation upon wounding and is induced by salicylic acid (O'Donnell et al., 1998), whereas *StSN2* did not. Expression pattern of *StSN2* also differs from that of Arabidopsis *WR3* gene, which is up-regulated by oligosaccharides (Titarenko et al., 1997b; Rojo et al., 1999), whereas *StSN2* was unaffected by chitosan treatment.

Responses to different pathogens and wound-inducible expression of the *StSN2* gene are in line with its putative defense role. The gene was induced in tubers infected with the *StSN2*-sensitive fungus *B. cinerea*, and repressed after inoculation with *R. so-*

lancearum and *E. chrysanthemi*. Furthermore, an *StSN2* expressed sequence tag (BG591412; Potato Genomic Project) has been recently associated with leaves infected with *Phytophthora infestans*. Up-regulation by fungal pathogens and down-regulation by bacterial ones have been described for other genes encoding plant antimicrobial peptides, such as *HvLTP4.3* from barley (*Hordeum vulgare*) and defensin *StPTH1* from potato (Moreno et al., 1994; Molina et al., 1996). Down-regulation of defense genes by some pathogens has been suggested as a mechanism to overcome plant defense (Jakobek et al., 1993; Wada et al., 1995). The antimicrobial properties of *StSN2* and its developmental and wound/pathogen-responsive expression patterns are congruent with a putative defense role. This function is also supported by the observed reduced virulence in planta of *R. solanacearum* and *E. chrysanthemi* mutants, which are sensitive to potato snakins (Titarenko et al., 1997a; Solanilla et al., 1998; *rfa*⁻ mutant in Table I). It should be noted that a defense role is not incompatible with the involvement of snakin/GASA genes in other plant processes and mechanisms (Herzog et al., 1995; Aubert et al., 1998; Kotilainen et al., 1999; Raventos et al., 2000).

MATERIALS AND METHODS

Biological Materials

Potato (*Solanum tuberosum* cv Jaerla) was cultivated in a growth chamber at 60% humidity at 28°C day and 22°C night, with a photoperiod of 14 h light/10 h dark. Bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus*, strain C5, *Ralstonia solanacearum*, strain K60 and *rfa*⁻ mutant (Titarenko et al., 1997a), *Erwinia chrysanthemi*, strain AC4150 (López-Solanilla et al., 1998), and *Rhizobium meliloti*, as well as the fungal pathogens *Botrytis cinerea* (strain 1) and *Fusarium solani* (strain 1), were from the Escuela Técnica Superior Ingenieros Agrónomos collection (Madrid). The fungal species *Aspergillus flavus*, *Bipolaris maydis*, *Fusarium culmorum*, *Colletotrichum lagenarium*, and *Colletotrichum graminicola* were from the Novartis Corp. collection (Research Triangle Park, NC). The fungus *Plectosphaerella cucumerina* was the gift of Dr. Brigitte Mauch-Mani (University of Fribourg, Switzerland), and the fungi *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *conglutinans* were kindly provided by Dr. María I.G. Roncero (Universidad de Córdoba, Spain).

Purification and Characterization of *StSN2*

A crude cell wall extract was obtained from frozen potato tuber material as previously described (Moreno et al., 1994; Segura et al., 1999) and subjected to reverse phase-HPLC on an Ultrapore C3 column (1 × 25 cm; 5- μ m particle; 300-Å pore; Beckman, San Ramon, CA). The linear gradient used was water (0.1% [v/v] trifluoroacetic acid)-2-propanol, 0% to 30% for 180 min and 30% to 50% for 15 min, at 0.5 mL min⁻¹. Fractions were collected by hand and

freeze dried. SDS-PAGE of the proteins was carried out in 12% (w/v) acrylamide gels following standard procedures. MALDI-MS was performed in the MALDI-II system from Kratos (Michigan, UK), using α -cyano (Sigma, St. Louis) as matrix and angiotensin I (MH, M_r 1,297.5) and cytochrome C (MH, M_r 12,361.5; M2H, M_r 6,182.2) as standards for mass calibration. Amino acid sequencing was carried out by automated Edman degradation of the intact protein.

Pathogen Inhibition Tests

Bacterial inhibition tests were carried out in sterile microtiter plates by mixing 50- μ L aliquots of bacterial suspension (final concentration 10^4 cfu mL⁻¹) in nutrient broth (Oxoid, Basingstoke, UK) with different amounts of the protein dissolved in 100 μ L of sterile water. After 16 to 24 h of incubation at 28°C, growth was recorded by measuring A_{490} in an ELISA plate reader. Fungal spores from 8-d-old cultures grown at 28°C on potato dextrose agar plates (Difco, Detroit, MI) were collected in sterile water and stored at -80°C in 20% (v/v) glycerol. Twenty-five microliters of a spore suspension (final concentration 10^4 spores mL⁻¹) in potato dextrose (Difco) was mixed in microtiter plates with different amounts of the protein dissolved in 50 μ L of sterile water. Plates were incubated at 28°C for 26 to 44 h and growth was recorded as above. Potato defensin StPTH1 and snakins StSN1 used in the inhibition experiments were purified as previously described (Moreno et al., 1994; Segura et al., 1999).

Characterization of StSN2

Total RNA extracted from potato tubers was used to obtain cDNA with the First-Strand Synthesis Kit (Amersham-Pharmacia, Rainham, UK) and an oligo(dT) primer. StSN2 cDNA was cloned by 3'-RACE using cDNA from potato tubers, an oligo(dT)-anchor primer from the kit indicated above, and two overlapping degenerate oligonucleotides corresponding to the amino acids sequences YKKIDCG and DCGGACA of the N-terminal region of the mature StSN2 peptide (PCR-annealing temperatures of 51°C and 56°C, respectively). The 5' region of the SN2 cDNA was cloned with the 5'-/3'-RACE Kit from Boehringer (Mannheim, Germany) using cDNA from potato tubers and the primers indicated in Figure 1C (PCR-annealing temperatures of 51°C and 54°C, respectively). Genomic sequences of StSN2 was amplified by PCR using specific primers deduced from their corresponding cDNAs (Fig. 1C) and potato genomic DNA obtained as described (Dellaporta et al., 1983). The PCR-amplified cDNAs and genomic fragments were cloned in the pGEM vector (Promega, Madison, WI), and the corresponding clones were sequenced using the ABIPrism Kit (Perkin-Elmer, Norwalk, CT).

Northern and Southern Blots

A StSN2 cDNA fragment (nucleotides 170–616) was randomly labeled with [³²P]ATP following standard procedures

(Sambrook et al., 1989) and used as a probe for northern and Southern experiments. Potato genomic DNA used in the Southern experiments was obtained as described (Dellaporta et al., 1983), digested with different endonucleases, subjected to electrophoresis on 0.8% (w/v) agarose gels, and transferred to Hybond N⁺ membranes (Amersham-Pharmacia) using standard procedures (Sambrook et al., 1989). RNAs were purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M LiCl (Lagrimini et al., 1987), and subjected to electrophoresis on 5% (v/v) formaldehyde/agarose gels. The gels were blotted to Hybond N⁺ membranes following standard procedures (Sambrook et al., 1989). RNA equal sample loads were checked by rehybridizing the blots with an 18S ribosomal cDNA probe of potato (X67238, nucleotides 361–959) obtained by PCR. Hybridization and washing of northern and Southern blots were carried out at 65°C according to Church and Gilbert (1984). Potato C17 probe was obtained by PCR amplification (nucleotides 851–1,216; Kirch et al., 1997) using potato genomic DNA as template. StPIN2 probe was kindly provided for Dr. Jose J. Sánchez-Serrano (Centro Nacional Biotecnología-Consejo Superior de Investigaciones Científicas, Madrid).

External Treatments

Four-week-old plants (five- to six-leaf stage) were used in all these experiments. ABA (100 μ M; Sigma) and GA (50 μ M GA₃; Sigma) were supplied in a phosphate solution (pH = 6.3) to excised potato plants, which were kept under the same growth conditions until the end of the experiments. For wounding experiment, expanded leaves were wounded perpendicular to the main vein with metallic forceps and material from the wounded leaves and non-damaged, upper leaves was harvested. Same-age leaves of non-wounded plants were used for controls. Salinity treatment was done by incubating excised potato plants in 250 mM NaCl, whereas control plants were incubated in a phosphate buffer (pH = 6.3). For drought treatment potato plants were removed from the pot and left to dry in the growth chamber. Chitosan treatment was done as described (Walker-Simmons and Ryan, 1984). Treatments with methyl jasmonate (50 μ M; Apex Organics, Leicester, UK; >90% pure), and the systemic acquired resistance activator benzo[1,2,3]-thiadiazole-7-carbothioic acid S-methyl ester (0.3 mM; Novartis, Basel) or its corresponding wettable powder alone, were done by spraying the whole plants with these compounds. Ethylene treatment was carried out by injecting 125 μ L L⁻¹ of the ethylene gas in a chamber containing excised potato plants incubated in water. In all these experiments, samples were collected at different times after treatment and frozen in liquid nitrogen.

Inoculation of Potato with Pathogens

Potato tubers were inoculated at three points with a suspension of *B. cinerea* spores (50 μ L of 2×10^5 spores mL⁻¹) by using a plastic tip containing the spores, which was inserted at a constant depth of 1.5 cm in the tubers.

Potato leaves of 4-week-old plants were inoculated in the upper side with four 20- μ L drops of a *B. cinerea* spore suspension (2×10^5 spores mL⁻¹). Inoculated plants and tubers were incubated at 28°C and 80% humidity. Tuber samples were collected by slicing the tuber and harvesting a disc (1-cm diameter and 2-cm height) of tissue around the inoculation points. Infection of potato tubers with *E. chrysanthemi* AC4150 strain (López-Solanilla et al., 1998) and *R. solanacearum* K60 strain (Titarenko et al., 1997a) were carried out as described above using 50 μ L of 10^7 cfu mL⁻¹ in 10 mM MgCl₂. Mock inoculations of tubers and leaves were done with 10 mM MgCl₂ alone. Samples were harvested as indicated above at different times after inoculation and frozen in liquid nitrogen.

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