Snakin: Structure, Roles and Applications of a Plant Antimicrobial Peptide

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Abstract: Snakins are plant antimicrobial peptides (AMPs) of the Snakin/GASA family, formed by three distinct regions: an N-terminal signal peptide; a variable site; and the GASA domain in the C-terminal region composed by twelve conserved cysteine residues that contribute to the biochemical stability of the molecule. These peptides are known to play different roles in response to a variety of biotic (i.e., induced by bacteria, fungi and nematode pathogens) and abiotic (salinity, drought and ROS) stressors, as well as in crosstalk promoted by plant hormones, with emphasis on abscisic and salicylic acid (ABA and SA, respectively). Such properties make snakin/GASA members promising biotechnological sources for potential therapeutic and agricultural applications. However, information regarding their tertiary structure, mode of action and function are not yet completely elucidated. The present review presents aspects of snakin structure, expression, functional studies and perspectives about the potential applications for agricultural and medical purposes.

Keywords: Snakin/GASA domain, cysteine-bridges, stress response, crosstalk.

1. INTRODUCTION

Along evolution plants developed complex defense mechanisms against a broad spectrum of pathogens. However, for many pathogenic microorganisms defense mechanisms are still inefficient, leading to disease development [1]. A common feature in defense strategies of plants and animals is the existence of a wide variety of small antimicrobial peptides (AMPs) that act as effector molecules of non-specific defense [2]. These peptides figure among most effective plant weapons, since they can be both constitutively expressed and stress induced by a wide range of biotic and abiotic stressors [3, 4]. Normally encoded by multigenic families, in which some genes are developmentally regulated, AMPs may act as potent broad-spectrum antibiotics, with potential as novel therapeutic agents.

Usually formed by 20 to 120 amino acid residues, these peptides can be rich in proline, glycine, histidine, arginine, tryptophan or cysteine [5]. Most of the cysteine-rich peptides have a globular structure, which is stabilized by disulfide bridges [6] and represent the major class of antimicrobial proteins found in plants. Among plant cysteine-rich AMPs, snakins are the most recently described peptides, isolated initially from potato and pepper, showing a broad spectrum activity against plant pathogens [7-9]. Studies based on sequence analysis, phenotypic characterization and expression pattern analysis have suggested that these peptides can be involved in diverse processes, both in development and in response to external factors [8, 10].

The present review focuses on the most significant features of this new family of antimicrobial peptides: structure, function, expression and potential applications, exploring its importance based on the current knowledge.

2. SNAKIN/ GASA FAMILY

2.1. Molecular Structure and Characterization

Snakin genes encode small proteins that comprise three distinct domains: (1) a signal peptide with 18-29 residues, (2) a variable region that is highly divergent between family members, both in amino acid composition and sequence length, and (3) the GASA domain, a conserved C-terminal region with approximately 60 amino acids, 12 of them are cysteine residues in conserved positions [10] (Fig. **1**).

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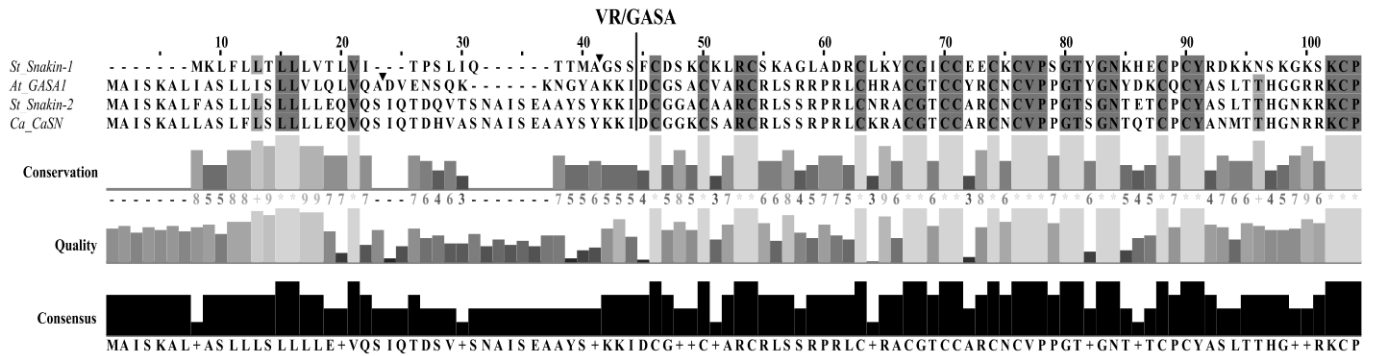
Fig. (1). Multiple sequence alignment of snakin/GASA peptides. The black triangles show de cleavage site for the signal peptide in *Solanum tuberosum* Snakin-1 (St-Snakin-1), *Arabidopsis thaliana* GASA1 (At_GASA1), *Solanum tuberosum* Snakin-2 (St_Snakin-2) and *Capsicum annuum* Snakin (Ca_CaSN), which is in the same position for At_Snakin-1, Ca_Casn and St_Snakin-2. The vertical bar indicates the limit between the variable region (VR) and the start of the GASA domain that exhibits twelve conserved cysteines and other nine conserved residues (dark gray in the alignment). Bars below the alignment indicate the levels of conservation, quality and, at the bottom, the consensus sequence.

Snakin/GASA (Gibberellin acid-stimulated from Arabidopsis) family comprises a group of widely distributed peptides among higher plants (Table **1**) sharing common structural features with many known antimicrobial peptides from eukaryotes, including the N-terminal putative signal sequence and a highly divergent acidic intermediate region. However, the main structural aspect is a cysteine-rich C-terminal signature, which contains 12 cysteine amino acids, in highly conserved positions (XCX3CX3CX8CX3CX2C CX2CXCX11CXCX12CX), forming a globular conformation, through six disulfide bonds (Fig. **1**). This C-Terminal region was described in *Arabidopsis thaliana* by Herzog *et al*. [10] and is called GASA domain. This arrangement is considered important for structure maintenance (specially the 3D conformation) and function of these peptides [11-15].

Studies have revealed that genes from this family may be regulated by plant hormones and participate in hormonal signaling pathways modulating the responses of the plant through their involvement in redox and hormone homeostasis. It is known that the presence of hormones such as Gibberellic Acid (GA) and SA has been associated to an increase in the expression level of snakin/GASA genes of *A. thaliana*, *Fagus sylvatica*, *Fragaria ananassa*, *Gerbera hybrida*, *Petunia hybrida*, *Oryza sativa* and *Zea mays* [10].

Genes of this family often encode a basic peptide with a molecular size below 11 kDa. After post-translational processes, the mature peptide basically consists of a structure with approximately 60 amino acid residues, with a molecular weight of approximately 7 KDa, for all snakin isolated so far. The basic isoelectric point (pI < 9.4) was described for Snakin-2 from potato and other GASA homologues, as well as for other antimicrobial peptides [7, 8, 16, 17], revealing that this feature is very conserved among AMPs and their homologues. Their basic character provides an advantage in interacting with negatively charged microbial membrane components [2]. All these features were also observed in a recently isolated snakin from a nematode-resistant pepper cultivar (*Capsicum annuum* cv. Santaka). According to Mao *et al*. [11] the CaSn (*C. annuum* snakin) gene encodes a protein of 104 amino acids residues (11 kDa) and a mature peptide of 66 amino acid residues that corresponds to a molecular mass of 7.03 kDa. Another member of this family was isolated from alfalfa (*Medicago sativa*), sharing many features (as a signal peptide comprising 25 residues) with the peptides of this group, representing a new member of the family [18].

Although there are three different genomic structures for Snakin/GASA members, only two structures were observed in snakins: (1) consisting of two exons and (2) consisting of three exons [11, 19]. Genes with two exons have been commonly described among antimicrobial peptides [6], including Snakin-1 from *Solanum tuberosum*, being considered a component of the constitutive defense against pathogens in both storage and reproductive plant organs [9, 20]. The second structure, also found in the Snakin-2, from potato [7] and in other AMPs [6] was associated with developmental stages and in response to specific pathogens, being considered an important component of both constitutive and induced defense [8, 21, 22]. The number of copies of Snakin-1 and Snakin-2 in the potato genome was estimated by southern blot hybridization, therefore the patterns observed indicated the existence of only one or two copies per genome [7, 9], whereas no CNV (Copy Number Variation) has been reported up to date.

Snakin/GASA proteins are expressed in different plant organs, however little is known about their 3D structure and mode of action [23]. To our knowledge, there is just one theoretical model for Snakin-1, whose putative structure is very similar to two other AMPs: thionins and α-helical hairpins [24, 25], formed by one short helix and two long α-helices. These findings strongly suggest that some classes of cysteine-rich AMPs share a common ancestor [6]. Furthermore, although the cysteine residues may be conserved in sequence and position, the disulfide bonds may be not structurally conserved, showing different patterns among snakin members [15, 26].

2.2. Synthesis, Developmental Process and Relationship to Stress Tolerance

2.2.1. Spatiotemporal Expression

When considering subcellular localization, there is a tendency among different snakin/GASA members to vary,

Table 1.   **Plant Snakin/GASA peptides previously described.**

Sequence name	Species	Family	References
GEG	*Gerbera hybrida*	Asteraceae	[52]
GASA1-14	*Arabidopsis thaliana*	Brassicaceae	[10, 11, 12, 36]
GmSN-like	*Glycine max*	Fabaceae	[Oliveira-Lima *et al.*]*
GsGASA	*Glycine soja*		[35]
MsSN1	*Medicago sativa*		[18]
Snakin-like	*Phaseolus vulgaris*		[28]
VuSN-like	*Vigna unguiculata*		[Oliveira-Lima *et al.*]*
FsGASA	*Fagus sylvatica*	Fagaceae	[53]
OsGASR1-2; OsGSR1	*Oryza sativa*	Poaceae	[54, 55]
ZmGSL1-10	*Zea mays*		[31]
FaGAST1	*Fragaria ananassa*	Rosaceae	[30]
CaSN	*Capsicum annuum*	Solanaceae	[8]
Gip1-5	*Petunia hybrida*		[56, 57]
RSI-1; GAST1	*Solanum lycopersicum*		[29, 34]
Snakin1-2	*Solanum tuberosum*		[10, 22]

* Author's data, paper *in prep*.

whereas some representatives (i.e., Snakin-1 from an agroin-filtrated *Nicotiana benthamiana*) had their localization experimentally observed in the plasmatic membrane. Moreover, depending on their structural features (for example, when a signal peptide is present) not all snakin/GASA proteins are secreted to the extracellular matrix possibly due to post-translational modifications. Other interactions may lead their localization as shown by Nahirñak *et al.* [27] reporting that Snakin-1 self-interacts *in vivo*. Another exemplified interaction regarded SN2-like from common bean (*Phaseolus vulgaris*) which associated with a proline-rich protein, resulting in a protein complex named FBCBP (French bean chitin-binding protein) [28].

Currently it is known that the expression of these peptides may vary for each gene and organ or may still be tissue specific. Most information about the function of some Snakin/GASA members was based on phenotypic characterization of mutants and transgenic plants or, still, on expression profiling. Some candidates seem to be involved in processes of arrest or promotion of cell elongation and division in adult plants, root formation (in early stages), flowering time and also fruit ripening [27, 29-33].

Among snakin genes, *Snakin-1* and *2* have different levels of expression along the age of the plant and developmental stage in potato plants. *Snakin-1* had its expression level decreasing along the plant development, being more active in young stages in tissues like carpels, petals, floral buds and tubers [9]. In turn, *Snakin-2* exhibited a pattern varying for both, development and tissues, being found in stems, sta-

mens, flower buds, leaves, shoot apices, carpels, petals and tubers, being also induced by pathogen infection [7].

2.2.2. Hormone Responsiveness

Many Snakin/GASA genes have been reported to have their transcription regulated by hormones [32, 34-36]. The first members of this family showing positive induction by GA and auxin hormones were the tomato *GAST1* (*Gibberellic Acid-Stimulated Transcript* 1) and *RSI-1* genes, respectively [29, 34]. Nevertheless, the positive induction by hormones (especially GA) was not considered a decisive factor for all members. For example, the transcription of potato *Snakin-2* was inhibited by GA, whereas the expression of *Snakin-1* of the same species had no alterations [7, 9]. On the other hand, under abscisic acid (ABA) treatment, the *Snakin-2* was induced, confirming the importance of crosstalk between snakin and this hormone, since ABA is an important wound signaling in Solanaceae species [7].

2.2.3. Redox Homeostasis

All identified members of the Snakin/GASA family presented a signature with twelve cysteine residues in the GASA domain, which is considered to exhibit a significant oxidative power due to their putative catalytic disulfide bonds (redox active cysteines), indicating a participation in redox regulation [15, 37, 38]. Besides, many metabolic pathways are activated by biotic stress which, in turn, trigger crosstalk responses, such as Reactive Oxygen Species (ROS) [10, 14, 39]. It is known that ROS is also involved in wounding where snakin may play a role in the redox regulation [10,

21]. Additionally, Nahirñak et al. [28] observed that metabolites that mitigate ROS effects were significantly reduced in Snakin-1 silenced strains from S. tuberosum in comparison to wild type. These lines also exhibited affected cell division, since the silenced lines presented smaller leaf sizes, what might reflect an inhibition of the cell division or growth, suggesting that Snakin-1 might be involved in cell division by modulating some metabolites [27].

2.2.4. Biotic and Abiotic Stress

As previously mentioned, snakins have been associated with a wide range of biotic and abiotic stresses [7, 14, 20, 21, 40, 41]. Interestingly, Snakin-1 was not induced in potato leaves by either biotic or abiotic stresses, indicating this peptide as a component of the plant constitutive defense [9]. In contrast to Snakin-1, Snakin-2 was positively regulated in potato leaves by wounding and presented a weak response to salinity stress, while drought stress caused no effect [7].

The following section details the existing evidence that Snakin genes are induced when plants are challenged by a wide range of microorganisms, whereas their overexpression in several angiosperms resulted in increased resistance against several microbial diseases. These findings, together with their reported in vitro antimicrobial activities may indicate a potential role of snakins to act as resistance traits against the main phytopathogens.

3. ROLE IN DEFENSE RESPONSE: IN VITRO AND IN VIVO BIOLOGICAL ACTIVITIES

Several in vitro and in vivo antimicrobial activities have been attributed to plant snakins. It has been reported that Snakin-1 (StSN1) and Snakin-2 (StSN2), isolated from S. tuberosum cv. Desireé and cv. Caerla, respectively, exhibit in vitro antimicrobial activity against important pathogens of potato and other plant species. StSN2 and StSN1 have been effective against the bacteria Clavivacter michiganensis subsp. sepedonicus and against fungal pathogens such as Fusarium solani, Colletotrichum lagenarium, Colletotrichum graminicola, Bipolaris maydis and Botrytis cinerea, among others, but not against Ralstonia solanacearum, an important potato bacterial pathogen [7, 9]. It was further observed that StSN1 presented an intermediate inhibitory effect on Listeria monocytogenes, a foodborne bacteria [42].

Additionally, StSN1 was heterologously expressed in Escherichia coli, followed by peptide purification and evaluation of its activity. This recombinant protein showed antibacterial and antifungal activity, inhibiting completely the growth of Clavibacter miciganensis subsp. sepedonicus and spore germination of Botrytis cinerea and Colletotrichum coccoides at a concentration of 14 mM [43], also inhibiting the growth of the mycelium of Gaeumannomyces graminis var. tritici - an important wheat pathogen [44]. A peptide of Capsicum annuum (CaSN), with high homology to StSN2 was also produced in E. coli. Free-living nematodes, like Caenorhabditis elegans were fed with E. coli strains expressing CaSN, leading to an inhibition of larval development, reduced growth and mobility, as well as death of most nematodes. In addition, plant-parasitic nematode intoxication was observed in toxicity assays using the supernatant containing the CaSN protein [8].

A possible role in planta has been suggested by overexpressing a snakin gene resulting in plants with increased resistance against several diseases. Transgenic lines of S. tuberosum subsp. tuberosum cv. Kennebec that accumulated high levels of SN1 mRNA from a potato wild relative (Solanum chacoense) showed heightened resistance against important potato pathogens, such as the fungus Rhizoctonia solani and the gram negative bacteria Erwinia carotovora [40] and also against Pectobacterium carotovorum subsp. carotovorum and Blumeria graminis [20]. Snakin-1 also decreased the virulence of the bacteria Dickeya chrysanthemi in sensitive tomato mutants [45], while the overexpression of a gene encoding SN2 increased tolerance to Clavibacter michiganensis subsp. michiganensis [21]. Transgenic wheat expressing StSN1 showed prominent resistance to 'take-all root rot', a wheat root disease caused by Gaeumannomyces graminis var. tritici [44], indicating a potential in vivo antifungal and antibacterial activity.

Plant snakins are induced by a wide range of biotic stresses and by plant hormones involved in stress signaling, also supporting their role in plant defense. In potato StSN1 gene activation was not observed when young leaves were subjected to biotic and abiotic stimuli [9]. However, a down-regulation of the gene encoding StSN2 was observed during Ralstonia solanacearum and Erwinia chrysanthemi infection, whereas an up-regulation was detected after infection with the compatible fungus Botrytis cinerea [7]. In Nicotiana benthamiana the silencing of Snakin-2 homologs increased susceptibility to Clavibacter michiganensis subsp. michiganensis [41], while their over-expression in transgenic tomato plants limited the invasiveness of this tomato pathogenic bacterium [21]. Variation regarding resistance and/or susceptibility to different pathogens indicates possible pathogen-specific associations or still induction mediated by other signaling molecules.

4. SNAKIN BIOTECHNOLOGY: STATE OF ART, APPLICABILITY AND PERSPECTIVES

Due to the identified antimicrobial activities [9] snakins have been considered interesting biotechnological targets. Thus, their biotechnological significance was indeed highlighted after the recognition of their involvement in the regulation of developmental processes in plants and also by the possibility to obtain considerable amounts of this peptide by expression in E. coli [10, 46]. Although their limited exploration in relation to other AMPs, the potential of snakins has been analyzed mainly by methods of transgeny and cisgeny (artificial gene transfer between organisms that could otherwise be conventionally bred).

In this regard, Almasia et al. [40] generated transgenic potato plants (S. tuberosum subsp. tuberosum cv. Kennebec) transformed via Agrobacterium tumefaciens with a construct encoding the gene SN1 of S. chacoense under the control of the CaMV 35S promoter. The resulting transgenic lines were evaluated under inoculation with Erwinia carotovora and Rhizoctonia solani, both relevant bacterial and fungal potato pathogens, respectively, whereas the transformed lines showed high rates of survival when compared to wild-type controls, indicating that StSN1 is involved in the resistance process against these pathogens.

In turn, Balaji & Smart [43] obtained genetically engineered tomato (*Solanum lycopersicum*) plants overexpressing *StSN2* or *ELP* (*Extensin like protein*) genes of the own species. The generated OGMs were evaluated under infection with the bacteria *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causal agent of bacterial wilt and canker, one of the most important tomato diseases throughout the world. In the transformed plants, the bacterial population was significantly reduced (100–10,000 fold) when compared to the wild type, indicating that StSN2 and ELP products limit the action of this pathogen, suggesting a potential *in vivo* antibacterial activity.

Additionally, Mohan *et al.* [22] obtained transgenic lines of potato (cv. Iwa) overexpressing the gene *Gibberellin Stimulated-Like 2* (*GSL2*; also known as *Snakin-2*) from the same species. Three trials performed independently showed that the modified strains exhibited a significant induction of *GSL2* transcriptional activity resulting in increased resistance to *Pectobacterium atrosepticum* (previously known as *Erwinia carotovora* subsp. *atroseptica*) the causal agent of potato blackleg, strengthening the role of this AMP in the defense against pathogens in potato.

Evaluations addressed to other environmental factors have shown that snakins may have a wide range of functions, highlighting their biotechnological potential. For example, Almasia *et al.* [47] characterized the promoter region (*PStSN1*) of the potato gene *StSN1*. Using bioinformatic approaches the authors identified 55 possible target regulation motifs associated to tissue specificity, abiotic stresses (high and low temperatures), defense and response to hormones, among others. To confirm the indicated functional dynamism the authors evaluated transgenic lines of *A. thaliana* expressing a reporter gene under control of the identified promoter (PStSN1:GUS). Contrary to expectations, there was no correlation between bioinformatic simulations (which indicated their potential functions) and the activation by the expected stressors, including phytohormones, light, dark, UV radiation or infection by *Pseudomonas syringae*. However, plants transformed with the identified promoter presented induction of responses associated to high or low temperatures, and mechanical damage (wounding), providing interesting targets for development of plants tolerant to extreme temperatures, insectivory [48], attack by pathogenic fungi [49] and viruses [50].

In addition to the antimicrobial role in biotic and abiotic stresses, recent data on snakins have increased the range of their potential biotechnological applications. Zare-Zardini *et al.* [51] observed that a protein named Snakin-Z presented, in addition to the already known antioxidant role, inhibitory effects over acetylcholinesterase (AChE) and butyryl-colinesterase (BChE) enzymes. This was considered a very important finding, since there are evidences that the inhibition of these enzymes may be applicable for the treatment of Alzheimer's disease, indicating that snakins may be a potential target for pharmacological trials in animal models to confirm these properties by *in vivo* essays [51].

The range of functions regarding snakins may still be underestimated, since several proteins bearing the Snakin/GASA domain play important roles in response to various types of stress. Due to the observed properties, snakins may have broad applications in different sectors: from agriculture, by obtaining tolerant/resistant transgenic plants to a range of stressful conditions; pharmaceutical industry, since their antifungal and antibacterial effects can be exploited for the development of antibiotics or antimicrobial substances, as well as a possible role in medicine, in the field of neurological diseases, due to its proposed ability to inhibit target enzymes involved in Alzheimer's disease [51]. Therefore, additional efforts are necessary to identify additional representatives of these interesting AMPs and their possible biological roles, especially considering their functional plurality.

CONCLUDING REMARKS

Among the diverse families of plant antimicrobial peptides, snakins deserve a special attention, considering that, at one side, they share many features with other AMPs and, by the other side, they possess peculiar features, like the number of cysteines (twelve) in the C-terminal region, which is the most important GASA domain signature, an important player of redox homeostasis.

Snakins seem to represent different roles in diverse plant developmental stages and tissues, from young to differentiated tissues, some induced by stress, exhibiting activity against a variety of bacteria, fungi and also nematodes. It has been also associated to hormones specially the GA, auxin and SA, being also constitutively expressed with participation in key events like cell division. Based on all these evidences, the broad potential of snakins is still to be explored, expanding the knowledge of their diversity, evolution, mode of action and its correlation with the structure, which constitute its main knowledge gap.

LIST OF ABBREVIATIONS

ABA	=	Abscisic acid
AMP	=	Antimicrobial peptide
CaMV	=	Cauliflower mosaic virus
CaSN	=	*Capsicum annuum* snakin
ELP	=	Extensin-like protein
FaGASA	=	Fragaria ananassa GASA
FBCBP	=	French bean chitin-binding protein
FsGASA	=	Fagus sylvatica GASA
GA	=	Gibberellic acid
GASA	=	Gibberellin acid-stimulated Arabidopsis
GAST	=	Gibberellic acid-stimulated transcript
GEG	=	Gerbera homolog of *GAST1* gene
Gip	=	GA-induced proteins
GmSN	=	*Glycine max* snakin
GsGASA	=	*Glycine soja* GASA
GSL	=	Gibberellin stimulated-like
MsSN	=	Medicago sativa snakin

OsGASR	=	*Oryza sativa* GA-stimulated transcript-related gene
ROS	=	Reactive oxygen species
RSI-1	=	Root system inducible-1
SA	=	Salicilic acid
StSN	=	*Solanum tuberosum* snakin
VuSN	=	*Vigna unguiculata* snakin
ZmGSL	=	*Zea mays* gibberellin-stimulated like

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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