Am. J. Potato Res.
DOI 10.1007/s12230-016-9494-8

Genome-wide Analysis of the Snakin/GASA Gene Family in *Solanum tuberosum* cv. Kennebec

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Abstract Snakin/GASA proteins have been involved in different aspects of plant growth and development although their exact role is still intriguing. All of them maintain 12 cysteines of the C-terminus in highly conserved positions that are responsible for their structure and are essential for their biochemical activity as antioxidants. Two members were isolated from *Solanum tuberosum* to date (Snakin-1 and Snakin-2) and were shown to have antimicrobial activity. We have recently demonstrated that Snakin-1 has additional roles in plant growth and development. We carried out a genome-wide search for new Snakin/GASA family members in potato. 16 Snakin/GASA genes were isolated, sequenced and characterized. Interestingly, we found in *Solanum tuberosum* subsp. *tuberosum* cv. Kennebec that Snakin-1, Snakin-2 and Snakin-3 expression is affected by bacterial and/or fungal inoculation. These results strengthen the participation of Snakin-1 and Snakin-2 genes in biotic stress tolerance and suggest that Snakin-3 is also involved in plant defense. The data presented here could be a good starting point for more focused and deep investigations regarding the biological functions of potato Snakin/GASA genes during plant development and in response to environmental stress.

Electronic supplementary material The online version of this article (doi:10.1007/s12230-016-9494-8) contains supplementary material, which is available to authorized users.

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Resumen A las proteínas Snakin/GASA se les ha involucrado en diferentes aspectos del crecimiento y desarrollo vegetal, aun cuando su papel exacto es intrigante. Todas mantienen doce cisteínas en el extremo C terminal, en posiciones altamente conservadas que son responsables de su estructura y son esenciales para su actividad bioquímica como antioxidantes. Se aislaron dos miembros de *Solanum tuberosum* a la fecha (Snakin-1 y Snakin-2) y se mostró que tienen actividad antimicrobiana. Recientemente hemos demostrado que Snakin-1 tiene funciones adicionales en el crecimiento y desarrollo de las plantas. Hicimos una búsqueda de amplitud genómica de nuevos miembros de la familia Snakin/GASA en papa. Se aislaron, secuenciaron y caracterizaron 16 genes Snakin/GASA. Interesantemente, encontramos en *Solanum tuberosum* subesp. *tuberosum* cv. Kennebec, que la expresión de Snakin-1, Snakin-2 y Snakin-3 es afectada por inoculación bacteriana y/o fúngica. Estos resultados refuerzan la participación de los genes de Snakin-1 y Snakin-2 en tolerancia biótica al estrés y sugiere que Snakin-3 también esta involucrada en la defensa de la planta. Los datos que aquí se presentan pudieran ser un buen punto de partida para investigaciones más enfocadas y profundas en relación a las funciones biológicas de los genes Snakin/GASA de la papa durante el desarrollo de la planta y en respuesta al agobio ambiental.

Keywords Snakin/GASA · Cysteine-rich domain · Potato · Plant development · Stress responses

Introduction

Snakin/GASA proteins have been classified into a large group of Cysteine-rich Peptides (CRPs) (Silverstein et al. 2007). The number and arrangement of the cysteine residues in the primary sequence distinguishes each CRP class from others such as

Published online: 28 January 2016

thionins, lipid transfer proteins, Snakin/GASA and defensins. Although the number and diversity of the CRPs characterized in plants is large, many more members of this superfamily may remain to be discovered (Silverstein et al. 2007). The Snakin/GASA genes encode small proteins in which three distinct domains can be defined: (1) a putative signal peptide of 18–29 residues; (2) a variable region displaying high divergence between family members both in aminoacid composition and sequence length; and (3) a C-terminal region of approximately 60 aminoacids with 12 cysteine residues in conserved positions named GASA domain (Aubert et al. 1998). None of the Snakin/GASA protein sequences possesses any known motif or active site and even though many of these genes were characterized and different biological functions were suggested, little is known about their mode of action. The fact that the number and positions of the cysteines have remained the same throughout evolution suggests that these residues play a central role (Ben-Nissan et al. 2004). In fact, they were shown to be essential for their biochemical activity as antioxidants (Rubinovich and Weiss 2010; Rubinovich et al. 2014). Moreover, three-dimensional structure prediction approaches revealed that they are involved in the formation of disulfide bonds responsible for protein folding (Porto and Franco 2013).

Expression analyses indicate that the spatial and temporal regulation of Snakin/GASA genes is highly specific. Although partially overlapping localizations between different members of the family have been described in different species, each of these genes displays a distinct expression pattern. Interestingly, most of them correlate with young tissues and actively growing organs (Taylor and Scheuring 1994; Ben-Nissan and Weiss 1996; Aubert et al. 1998; Kotilainen et al. 1999; Segura et al. 1999; Ben-Nissan et al. 2004; de la Fuente et al. 2006; Roxrud et al. 2007; Zhang and Wang 2008; Wang et al. 2009; Almasia et al. 2010; Zimmermann et al. 2010). Furthermore, most of these genes are regulated by plant hormones and participate in hormonal signaling pathways modulating hormonal responses and levels (Shi et al. 1992; Taylor and Scheuring 1994; Herzog et al. 1995; Kotilainen et al. 1999; Segura et al. 1999; Bouquin et al. 2001; Berrocal-Lobo et al. 2002; Ben-Nissan et al. 2004; de la Fuente et al. 2006; Furukawa et al. 2006; Zhang and Wang 2008; Alonso-Ramírez et al. 2009; Wang et al. 2009; Zhang et al. 2009; Zimmermann et al. 2010; Nahirñak et al. 2012; Nahirnak 2015). According to homology analyses, phenotypic characterization of transgenic plants and expression pattern studies, Snakin/GASA peptides could be involved in a variety of plant development processes and plant environmental responses. Some of these peptides have been implicated in cellular processes such as promotion of cell elongation (Shi et al., 1992; Ben-Nissan and Weiss 1996; Ben-Nissan et al. 2004), cell elongation arrest (Kotilainen et al. 1999; de la Fuente et al. 2006) and cell division (Aubert et al. 1998; Ben-Nissan et al. 2004; Furukawa et al. 2006; Nahirñak et al. 2012). Moreover, Snakin/GASA proteins have been involved in root formation, stem growth, flowering time and fruit ripening (Taylor and Scheuring 1994; Ben-Nissan et al. 2004; de la Fuente et al. 2006; Zhang et al. 2009; Zimmermann et al. 2010; Moyano-Canete et al. 2013). In addition, some members of this family participate in pathogen defense (Segura et al. 1999; Berrocal-Lobo et al. 2002; Almasia et al. 2008; Kovalskaya and Hammond 2009; Faccio et al. 2011; Mao et al. 2011; Balaji and Smart 2012; Rong et al. 2013; Mohan et al. 2014), as well as heat, salt or oxidative stress tolerance (Ko et al. 2007; Alonso-Ramírez et al. 2009; Sun et al. 2013).

The tomato Gibberellic Acid-Stimulated Transcript 1 gene (*GAST1*) was the first member of the Snakin/GASA family to be described in an attempt to identify gibberellic acid (GA) regulated genes in shoots of the GA-deficient *gib* mutant (Shi and Olszewski 1998). Since then, numerous members of this family have been identified in a wide range of species; however, only a small number of these genes have been characterized for most of the studied species.

Recent advances in genome-scale sequencing technologies provide new tools to identify novel genes. In this context, Snakin/GASA has been shown to represent a large gene family in some species: to date 15 Snakin/GASA genes have been identified in *Arabidopsis thaliana* (Zhang and Wang 2008), nine in rice and ten in maize (Zimmermann et al. 2010). The identification of Snakin/GASA genes in distantly related species highlights the importance of these proteins and suggests that they fulfill essential functions in plants.

In potato, two members of the Snakin/GASA family have been isolated to date (Snakin-1 and Snakin-2) and both have antimicrobial activity (Segura et al. 1999; Berrocal-Lobo et al. 2002; Almasia et al. 2008; Faccio et al. 2011; Balaji and Smart 2012; Rong et al. 2013; Mohan et al. 2014). Recently we have demonstrated that *Snakin-1* partial silencing affects cell division, leaf metabolism and cell wall composition; which suggests that it has additional roles in plant growth and development (Nahirñak et al. 2012). More recently, Meiyalaghan et al. reported that the antisense knock-down expression of these Snakin/GASA genes was not possible (Meiyalaghan et al. 2014). Even though this is contradictory with our previous result they proposed that this could be due to the different promoters used in these studies. In our work using 35S promoter we obtained an abnormal phenotype; while Meiyalaghan et al. used the Lhca3 promoter, which confers higher and more stable transgene expression than the 35S, failed to recover viable plants. Consequently, the authors suggest that the lethality observed using the Lhca3 promoter is not unexpected given the dramatic phenotypes observed with the partial silencing from the use of the 35S promoter. The observed lethality reinforces the essential role of Snakin/GASA gene family in potato development.

In this study, 16 Snakin/GASA genes from potato were isolated, sequenced and characterized. We describe for the first time detailed information about the genomic structures,

phylogeny and conserved motifs/*cis*-elements in their promoter sequences. Since previous studies revealed some differences in the expression pattern of Snakin/GASA genes analyzed in different varieties (Segura et al. 1999; Berrocal-Lobo et al. 2002; Ben-Nissan et al. 2004; Almasia et al. 2008; Potato Genome Sequencing et al. 2011; Meiyalaghan et al. 2014), expression analyses of Snakin/GASA genes in *Solanum tuberosum subsp. Tuberosum* cv. Kennebec are also presented in order to improve our understanding of their functions.

Materials and Methods

Identification of Predicted Genes That Encode Snakin/GASA Proteins

Snakin/GASA genes were searched in the Potato Genome Sequencing Consortium (PGSC) database (http://solanaceae.plantbiology.msu.edu/integrated_searches.html) using the InterPro GASA domain annotation (IPR003854; http://www.ebi.ac.uk/interpro/IEntry?ac=IPR003854) as query. The potato genome database of SGN (http://solgenomics.net) was also used in this analysis. For Snakin/GASA gene cloning, the PCR products were obtained from *Solanum tuberosum subsp. Tuberosum* cv. Kennebec total DNA (the primers for the PCRs reaction are listed in Supplemental Table S1). They were separated in 1.5 % agarose gel, purified with QIAquick Gel Extraction Kit (Qiagen), cloned into pGEM-T vector (Promega Corporation, USA) and sequenced with vector specific primers. The cloned sequences were analyzed by the BioEdit Sequence Alignment Editor and multiple sequence analyses were performed by the ClustalW software package (http://www.ebi.ac.uk/clustalw/). The sequences were deposited in NCBI (GenBank accessions numbers: PGSC0003DMG400014441: KP293878, PGSC0003DMG400018474: KP293879, PGSC0003DMG400025759: KP293880, PGSC0003DMG400003641: KP293881, PGSC0003DMG400003642: KP293882, PGSC0003DMG400009244: KP293883, PGSC0003DMG400015602: KP293884, PGSC0003DMG400024474: KP293885, PGSC0003DMG400029338: KP293886, PGSC0003DMG402015689: KP293887, PGSC0003DMG400001226: KP293888, PGSC0003DMG400001227: KP293889, PGSC0003DMG400007621: KP293890, PGSC0003DMG401019533: KP293891, PGSC0003DMG400033044: KP293892, PGSC0003DMG401001384: KP293893, PGSC0003DMG400001598: KP293894. The location of Snakin/GASA genes on potato chromosomes and the gene structure were obtained from the PGSC whole genome annotation data.

Sequence and Phylogenetic Analyses of Snakin/GASA Proteins

Protein sequences were aligned by the ClustalW software package. Phylogenetic trees were constructed by the Neighbor-Joining method, with 1000 bootstrap replicates, according to the Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 (http://www.megasoftware.net/mega.html). The proteins and their accession numbers used for alignment and phylogenetic tree construction are as follows: Snakin-1 [GenBank: EF206292.1]; PGSC0003DMP400025440; PGSC0003DMP400044609; PGSC0003DMP400032186; Snakin-2; PGSC0003DMP400006510, PGSC0003DMP400016314, PGSC0003DMP400042322, PGSC0003DMP400051082, PGSC0003DMP400027335.

PGSC0003DMP400006509, Snakin-3, PGSC0003DMP400002225, PGSC0003DMP400002223, PGSC0003DMP400055795, PGSC0003DMP400013485, PGSC0003DMP400033943, PGSC0003DMP400002503, GEG [GenBank: CAB45241]; GAST1 [GenBank: 004,232,126]; RSI-1 [GenBank: 001,234,666]; GIP1 [GenBank: CAA60677]; GIP2 [GenBank: AAG43509]; GIP3 [GenBank: CAD10104; GIP4 [GenBank: CAD10105:]; GIP5 [GenBank: CAD10106]; FaGAST1 [GenBank: AAB97006]; FaGAST2 [GenBank: XP_004290947]; FsGASA[GenBank: CAJ77893]; GsGASA1 [GenBank: ADX36135]; CaSn [GenBank: ACC91329]; OsGSR1 [GenBank: AAT42201]; OsGASR1 [GenBank: NP_001051348]; OsGASR2 [GenBank: NP_001053024]; OsGSL1 [GenBank: NP_001055385]; OsGSL5 [GenBank: NP_001055630]; OsGSL7 [GenBank: NP_001050637]; OsGSL8 [GenBank: NP_001058649]; OsGSL9 [GenBank: EAZ40506]; OsGSL10 [GenBank: NP_001063170]; GASA1 [GenBank: AAM96972]; GASA2 [GenBank: NP_192699]; GASA3 [GenBank: NP_192698]; GASA4 [GenBank: NP_197027]; GASA5 [GenBank: NP_566186]; GASA6 [GenBank: NP_177605]; GASA7 [GenBank: NP_179096]; GASA8 [GenBank: NP_181486]; GASA9 [GenBank: NP_173683]; GASA10 [GenBank: NP_568914]; GASA11 [GenBank: NP_179433]; GASA12 [GenBank: NP_180639]; GASA13 [GenBank: NP_196996]; GASA14 [GenBank: NP_001077504]; GASA15 [GenBank: NP_001078130]; PRGL [GenBank: AAS48461]; GhGASL1–GhGASL7 [GenBank: KC147715–KC147721].

Promoter Region Analysis of Snakin/GASA Genes

To investigate the presence of *cis*-elements in promoter sequences of potato Snakin/GASA genes, we analyzed 1500 bp of genomic sequences upstream of the initiation codon from the PGSC database using the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/

plantcare/html/) (Lescot et al. 2002). Only 800 bp for *Snakin-2* (*PGSC0003DMG400001598*) were used for this analysis because of the unavailability of the upstream full DNA sequence in PGSC database.

Northern Blot and qRT-PCR

For *Northern blot* analyses, RNA was isolated using RNAquous kit (Ambion) and 10 μg was electrophoretically resolved in 1.5 % agarose gels with MOPS buffer, transferred to Hybond N+ Nylon Membrane™ (Amersham) for 16 h and crosslinked under a UV lamp. Probes were synthesized and labelled with α-[32P]-dCTP by the Prime-a-Gene labeling System (Promega). Hybridizations were performed for 14 h at 42 °C with ULTRAhyb[a] hybridization solution (Ambion). Membranes were washed twice in 2X SSC–0.1 % SDS for 10 min at 42 °C, once in 1X SSC–0.1 % SDS for 20 min at room temperature and once in 0.1X SSC–0.1 % SDS for 10 min at room temperature. Membranes were exposed overnight and scanned by a Typhoon scanner (Amersham Biosciences).

For qRT-PCR, RNA was extracted with the RNAquous kit (Ambion) and treated with DNase (Invitrogen). Reverse transcription reactions were carried out with SuperScript III (Invitrogen) and random primers. qRT-PCR was performed by an ABI PRISM 7500 (AppliedBiosystems) with SYBR Green master mix (Qiagen). Three biological and technical replicates for each gene were run. qRT-PCR data analyses and primer efficiencies were obtained with LinRegPCR software (Ramakers et al. 2003). Potato elongation factor 1α (Ef1α) was used as internal control (Nicot et al. 2005). Relative expression ratios and statistical analysis were performed by fgStatistics software interface (http://sites.google.com/site/fgStatistics/) using the algorithm developed by Pfaffl and associates (Pfaffl et al. 2002). Baseline expression pattern is indicated by the red line in each graph. Red lines correspond to expression level of mock treated plants in the corresponding time point except to spatial expression studies where red line correspond to expression in root.

All primer sets are listed in Table S1.

Biological Material, Growth Conditions and External Treatments

Solanum tuberosum spp. tuberosum cv Kennebec plants were grown in a greenhouse at 18–25 °C, under a 10 h dark-14 h light cycle, in 4.72 in. in diameter pots.

For spatial expression analyses, samples were taken from different tissues of 8-week-old plants.

Hormone treatment: Treatments with abscisic acid (100 μM; Sigma) and GA (100 μM; Sigma) were performed by spraying 6-week-old plants according to Segura et al. 1999. Control plants were mock-inoculated with the same solution without the corresponding hormone. Samples were collected at 6, 12, and 24 h after treatment based on Berrocal-Lobo et al. 2002.

Bacterial strains and infection: P. *syringae* pv. *tabaci* strains were cultured overnight in King's B medium supplemented with 25 mg L^{-1} rifampicin and 2 mg L^{-1} tetracycline at 28 °C with 200 rpm shaking and subsequently they were centrifuged. Bacterial pellets were resuspended in 10 mM $MgCl_2$ and adjusted to 10^8 CFU/ml. Fully expanded leaves of 6-week-old potato plants were inoculated with bacteria or 10 mM $MgCl_2$ (mock-inoculated plants) using a needleless hypodermic syringe. Leaf samples were collected at 6 and 24 h after inoculation.

Rhizoctonia solani in vitro inoculation: A PDA block (1 cm^2) with *Rhizoctonia solani* mycelium was placed in the centre of a magenta box carrying 4 plants aseptically grown for 4 weeks in the magenta corners (equidistant from the centre). After 7 days the mycelia started to reach the plants and by 12 days after the disc inoculation the mycelia had reached the plants completely. Whole-plant samples were harvested at 7, 10 and 12 days after inoculation.

Results

Isolation, Sequencing, Chromosomal Localization and Structural Analyses of Potato Snakin/GASA Genes

In this study, 16 Snakin/GASA family members were isolated and sequenced from potato in addition to the previously reported genes (*Snakin-1* and *Snakin-2*). This finding confirms that the potato Snakin/GASA gene family consists of at least 18 members (Fig. 1). These genes were numbered according to the nomenclature established in the potato genome database: PGSC corresponds to Potato Genome Sequencing Consortium; 0003 represents the third version of the genome assembly; DM indicates the doubled monoploid used (*S. tuberosum* group Phureja DM1-3 516 R44); and G, T or P refers to gene, transcript or protein respectively. Chromosome localization study of potato Snakin/GASA genes indicated that all of them correspond to different loci in the genome and that they are distributed on nine of the 12 potato chromosomes (Fig. 1). Using the Potato genome database, we obtained the complete genomic sequence of these genes and subsequently designed specific primers. The identified Snakin/GASA family genes were further confirmed by PCR amplification, cloning and sequencing from the commercially cultivated *S. tuberosum* subs*p. Tuberosum* cv. Kennebec genotype (Supplemental file S2). Even though it would be interesting to search for allelic variations, this was not the focus of our work and we present here the consensus sequences (DNA sequences determined in both directions from at least 5 independent clones of each gene). The coding

	Name	Gene Transcript Protein	Chromosome	Protein length (aa)	Signal Peptide (aa)
Snakin-1 →	Snakin-1	PGSC0003DMG400021517 PGSC0003DMT400055426 PGSC0003DMP400037307	4	88	25
	GAST1 protein	PGSC0003DMG400014441 PGSC0003DMT400037423 PGSC0003DMP400025440	12	88	25
	GAST1 protein	PGSC0003DMG400025759 PGSC0003DMT400066182 PGSC0003DMP400044609	1	89	26
	GAST1 protein	PGSC0003DMG400018474 PGSC0003DMT400047535 PGSC0003DMP400032186	8	89	18
Snakin-2 →	Snakin-2	PGSC0003DMG400001598 PGSC0003DMT400004046 PGSC0003DMP400002893	1	104	23
	Gast1	PGSC0003DMG400003642 PGSC0003DMT400009368 PGSC0003DMP400006510	2	102	23
	Gibberellin-regulated protein 1	PGSC0003DMG400009244 PGSC0003DMT400023914 PGSC0003DMP400016314	11	103	22
	Gonadotropin beta chain	PGSC0003DMG400024474 PGSC0003DMT400062877 PGSC0003DMP400042322	3	143	18
	GAST	PGSC0003DMG400029338 PGSC0003DMT400075426 PGSC0003DMP400051082	12	113	24
	Gibberellin-regulated protein 3	PGSC0003DMG400015602 PGSC0003DMT400040298 PGSC0003DMP400027335	5	90	22
	Gast1	PGSC0003DMG400003641 PGSC0003DMT400009367 PGSC0003DMP400006509	2	103	23
Snakin-3 →	Protein RSI-1	PGSC0003DMG402015689 PGSC0003DMT400040585 PGSC0003DMP400027523	11	96	29
	Gibberellin regulated protein	PGSC0003DMG400001227 PGSC0003DMT400003089 PGSC0003DMP400002225	6	94	19
	Gibberellin-regulated family protein	PGSC0003DMG400001226 PGSC0003DMT400003087 PGSC0003DMP400002223	6	94	19
	Gip1	PGSC0003DMG400033044 PGSC0003DMT400083077 PGSC0003DMP400055795	6	103	24
	GASA2	PGSC0003DMG400007621 PGSC0003DMT400019726 PGSC0003DMP400013485	4	114	24
	Gip1	PGSC0003DMG401019533 PGSC0003DMT400050285 PGSC0003DMP400033943	3	104	24
	GAST1	PGSC0003DMG401001384 PGSC0003DMT400003510 PGSC0003DMP400002503	2	112	25

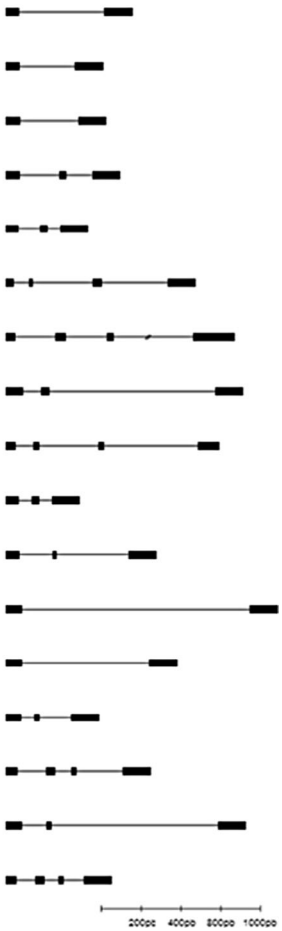


Fig. 1 Identification of Snakin/GASA gene family members in potato. The putative signal peptide was predicted by the SignalP.v4.0 software. Right part illustrates the exon–intron organization of corresponding genes. The exons and introns are represented by boxes and lines, respectively

sequences of the potato Snakin/GASA genes ranged from 472 bp (*PGSC0003DMG400003641*) to 2584 bp (*PGSC0003DMG400024474*). The comparison of the published transcript sequences with the corresponding genomic DNA sequence showed that the coding sequences of all Snakin/GASA genes are disrupted by one to three introns (Fig. 1).

The sequence analysis of the potato Snakin/GASA predicted proteins showed that the three distinct domains characteristics of this family are found in all of them. Particularly for the *PGSC0003DMG400015602* gene, protein predictions based on the published potato genomic sequence revealed a putative gene product of 90 amino acid residues which corresponds to an incomplete GASA domain. However, in *S. tuberosum* subs*p. Tuberosum* cv. Kennebec genotype we found that the predicted protein might be of 106 amino acid residues and it presents a full GASA domain. This difference is due to a base pair change in the annotated sequence that generated a putative premature stop codon.

All of the potato Snakin/GASA family members contain an individual stretch of 18 to 29 amino acids at the N terminus, which, according to the SignalP.v4.0 software, is a cleavable signal sequence characteristic of proteins secreted into the extracellular matrix. Downstream of the putative signal sequence, the Snakin/GASA predicted proteins contain a region displaying high divergence between family members. This region is variable in amino acid composition and ranges in sequence length from only four residues in PGSC0003DMP400037307, PGSC0003DMP400025440 and PGSC0003DMP400044609 to 62 in PGSC0003DMP400042322. Finally, 20 amino acids, including the 12 cysteines, are perfectly conserved within the C-terminal domain of 59 to 64 amino acids. A multiple sequence alignment illustrates the conservation of this domain (Fig. 2). The total size of these proteins

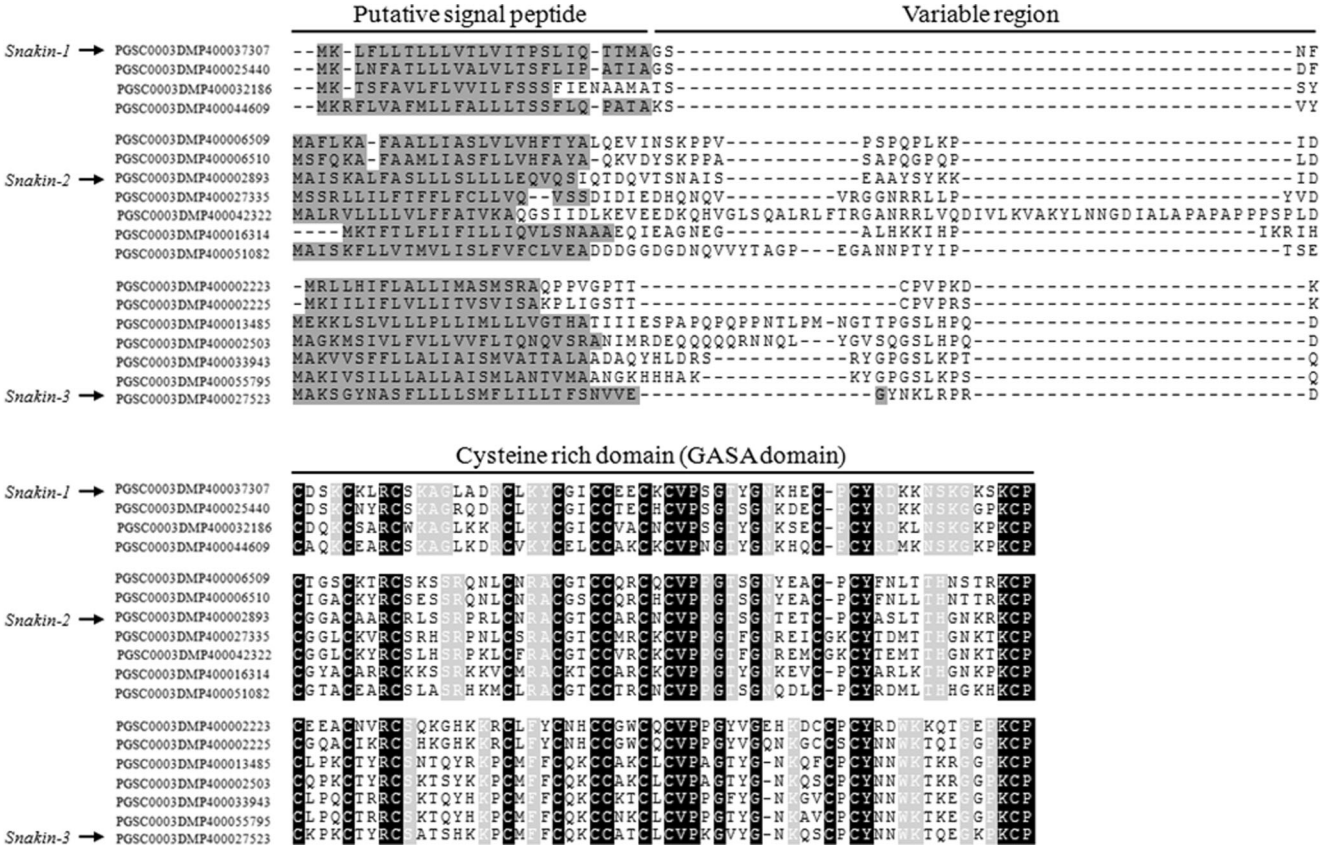


Fig. 2 Multiple sequence alignment of predicted potato Snakin/GASA proteins. Conserved aminoacids across all the family members are black shaded, whereas highly conserved residues that are relevant for subfamily classification are gray shaded. Dark gray shading indicates the putative signal peptide according to the SignalP.v4.0 software

varied from 88 amino acids (PGSC0003DMP400037307 and PGSC0003DMP400025440) to 143 amino acids (PGSC0003DMP400042322) (Fig. 1).

Phylogenetic Analysis of Snakin/GASA Proteins

To investigate the relationships among potato Snakin/GASA proteins, the full-length putative protein sequences were used to build a phylogenetic tree (consensus sequences of Kennebec were used for the Fig. 3 and similar results were obtained using sequences generated by PGSC). The Snakin/GASA proteins in potato could be grouped into three subfamilies (I–III) in agreement with the classification previously proposed by Berrocal-Lobo et al. (Berrocal-Lobo et al. 2002). An amino acid sequence alignment and a phylogenetic tree analysis including Snakin/GASA proteins from other species resulted also in three major groups; which is consistent with previous studies (Zimmermann et al. 2010) (Fig. 3).

Promoter Region Analysis of Potato Snakin/GASA Genes

Even though the exact role played by Snakin/GASA proteins is still intriguing, they have been involved in different aspects of plant development and stress tolerance. Putative promoter sequences (1.5-kb upstream of the start codon except for *Snakin-2*) were obtained from the potato genome sequence and the presence of *cis*-regulatory elements was examined with the database of Plant *cis*-acting Regulatory DNA Elements (PlantCARE) (Lescot et al. 2002). *In silico* analyses revealed that potato Snakin/GASA gene upstream sequences carry a variety of potential *cis*-acting elements (Supplemental file S3). Among these elements, there are binding sites for transcription factors that are regulated by hormones, such as abscisic acid (ABA), gibberellic acid (GA), auxin, jasmonic acid, salicylic acid and ethylene or by stress responses (such as pathogen defense, heat and low temperature stress, drought). As shown in Table 1, the patterns of *cis*-acting elements differed among the potato Snakin/GASA genes. Remarkably, the *PGSC0003DMG400014441* gene promoter lacked hormones response elements, whereas *PGSC0003DMG401019533* lacked stress response elements.

Regulation of Potato Snakin/GASA Gene Expression

Given that analyses of gene regulation can provide information about their functions *in planta*, we analyzed the expression in different organs of some members of each subfamily of Snakin/GASA genes in potato plants. It has been reported that

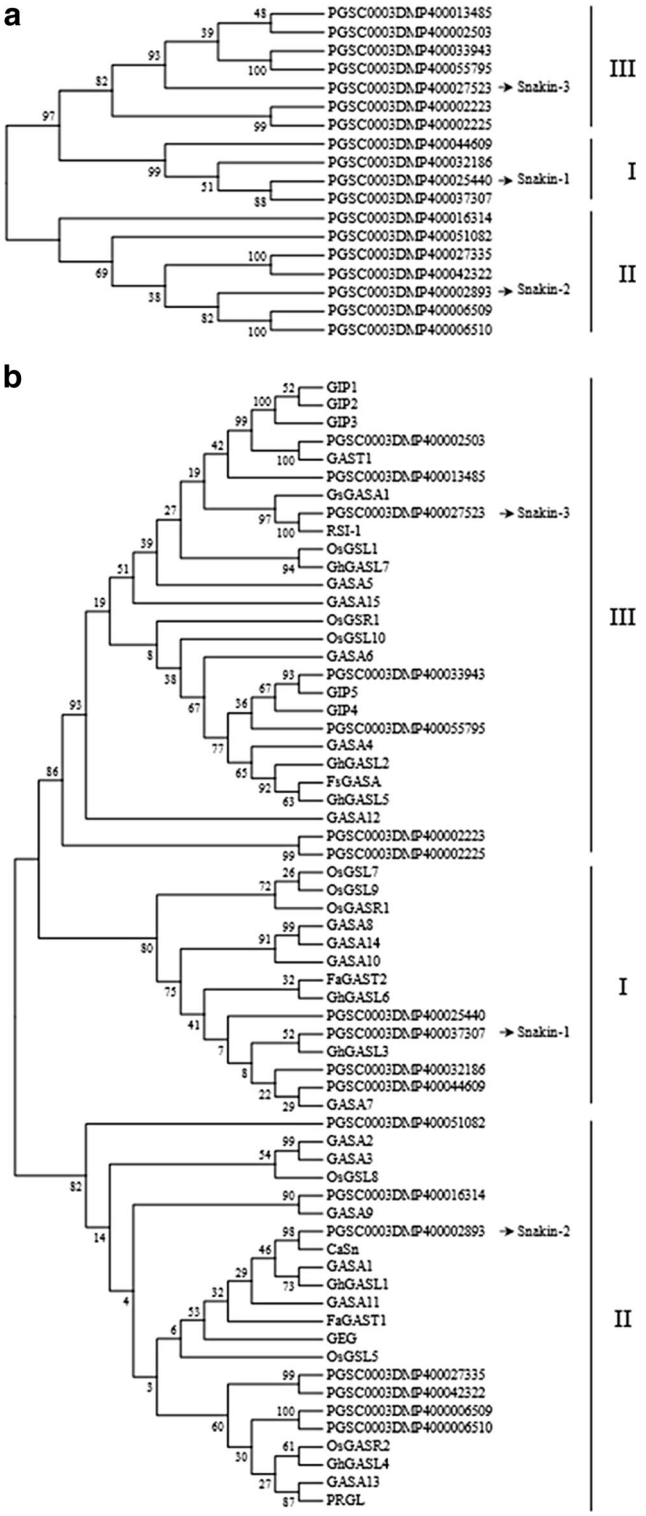


Fig. 3 Phylogenetic analysis of predicted Snakin/GASA proteins. An unrooted phylogenetic tree constructed by Neighbor-joining. The branches were reconfirmed by 1000 bootstrap resamplings. The bootstrap value of each branch is indicated. (a) Phylogenetic relationships among Snakin/GASA proteins in potato. (b) Phylogenetic tree constructed from the multiple-sequence alignment of Snakin/GASA proteins reported from different plant species. I, II, III: Subfamily I, Subfamily II and Subfamily III

Snakin-1 and *Snakin-2* exhibit a distinct expression pattern (Segura et al. 1999; Berrocal-Lobo et al. 2002). Since we study a different genotype from the ones previously analyzed (*Solanum tuberosum* cv. Desireé for *Snakin-1* and *Solanum tuberosum* cv Jaerla for *Snakin-2*), we evaluated the spatial expression pattern of *Snakin-1*, *Snakin-2* and also *Snakin-3* by *Northern blot* and qRT-PCR using specific primers in different tissues of *Solanum tuberosum subsp. Tuberosum* cv. Kennebec plants. These analyses showed a tissue specific expression pattern for each member and also some differences from the previously reported data for *Snakin-1* and *Snakin-2*. The expression of *Snakin-1* was particularly high in axillary buds and stems and, in contrast to Segura et al. (Segura et al. 1999), we also detected it in leaves. The *Snakin-2* expression was high in tubers, flowers and leaves and, in contrast to previous data (Berrocal-Lobo et al. 2002), we also found that this gene was highly expressed in roots. Finally, we found that *Snakin-3* was expressed in roots, stolons, stems and axillary buds (Fig. 4). Similar results were obtained by both techniques although qRTPCR was more sensitive, as expected. In addition, to study if different members of the same subfamily share a similar expression pattern, the spatial regulation of other members of each subfamily was studied by qRT-PCR: Results indicate a particular spatial expression pattern for each Snakin/GASA gene, for example: *PGSC0003DMG400024474* (Subfamily II) was mainly expressed in tuber, stem, axillary bud and shoot apex; *PGSC0003DMG400003641* (Subfamily II) in roots and flowers; *PGSC0003DMG4000092444* (Subfamily II) in tubers and *PGSC0003DMG4000015602* (Subfamily II) in flowers.

As we mentioned before, most of Snakin/GASA genes are regulated by plant hormones and external factors. In agreement, in silico analyses revealed that potato Snakin/GASA genes upstream sequences carry a variety of hormones and stress response elements (Table 1). In this context, we first analyzed the effect of GA and ABA on the expression pattern of *Snakin-1*, *Snakin-2* and *Snakin-3* genes in different tissues. We found that *Snakin-1* expression was downregulated by GA 24 h after treatment in shoot apex, 12 h and 24 h after treatment in leaf and in 6 h and 12 h after treatment in stem ($p < 0$, 05) and it seemed to be unaltered by ABA in shoot apex, leaf or stem. The regulation of *Snakin*-2 appeared to be more complex. It was induced by GA 12 h and 24 h after treatment in shoot apex and it was repressed by GA 24 h after treatment in leaf. In addition, the *Snakin*-2 gene expression was induced by ABA 12 h after treatment in the shoot apex. In a different way, *Snakin-3* expression remained unaltered with GA and it was downregulated by ABA 24 h after treatment in stem (Fig. 5).

Finally, we also investigated the effects of pathogen infection on the expression of these potato Snakin/GASA genes. Leaves were inoculated with P. *syringae* pv. *tabaci* and samples were taken at 6 h and 24 h after infection. We observed

Table 1 Hormone and stress response motifs identified in the putative promoter sequences. Numbers of *cis*-regulatory elements identified along the putative promoter sequences of each potato *Snakin*/GASA gene using the database of the Plant *cis*-acting Regulatory DNA Elements (PlantCARE)

	Site Name	Sequence	Function	*Snakin-1*	*PGSC0003DMG400018474*	*PGSC0003DMG400014441*	*PGSC0003DMG400025759*	*Snakin-2*	*PGSC0003DMG400003641*	*PGSC0003DMG400003642*	*PGSC0003DMG400009244*
HORMONES	AuxRR-core	GGTCCAT	cis-acting regulatory element involved in auxin responsiveness	1							
	TGA-box	TGACGTAA	part of an auxin-responsive element				1				
	TGA-element	AACGAC	auxin-responsive element							1	
	CGTCA-motif	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness	2	1		2			1	
	TGACG-motif	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness	2	1		2			1	
	ERE	ATTTCAAA	ethylene-responsive element	3			1	1			
	GARE-motif	AAACAGA	gibberellin-responsive element	1			1		2		
	GARE-motif	TCTGTTG	gibberellin-responsive element								
	TATC-box	TATCCCA	cis-acting element involved in gibberellin-responsiveness								
	P-box	CCTTTTG	gibberellin-responsive element		1					1	2
	P-box	GCCTTTTGAGT	gibberellin-responsive element				1				1
	TCA-element	GAGAAGAATA	cis-acting element involved in salicylic acid responsiveness	1	1		1				
	TCA-element	TCAGAAGAGG	cis-acting element involved in salicylic acid responsiveness				1				
	TCA-element	CCATCTTTTT	cis-acting element involved in salicylic acid responsiveness						1	1	
	TCA-element	GAGAAGAATA	cis-acting element involved in salicylic acid responsiveness								
	ABRE	CCTACGTGGC	cis-acting element involved in the abscisic acid responsiveness		6		1				
	ABRE	TACGTG	cis-acting element involved in the abscisic acid responsiveness				1	1			
	ABRE	GCAACGTGTC	cis-acting element involved in the abscisic acid responsiveness							1	
			TOTAL	10	10	0	12	2	3	6	3
STRESS	ARE	TGGTTT	cis-acting regulatory element essential for the anaerobic induction	2			1		1	2	2
	GC-motif	CCCCCG			2					1	

Table 1 (continued)

	Site Name	Sequence	Function	*Snakin-1*	*PGSC0003DMG400018474*	*PGSC0003DMG400014441*	*PGSC0003DMG400025759*	*Snakin-2*	*PGSC0003DMG400003641*	*PGSC0003DMG400003642*	*PGSC0003DMG400009244*
			enhancer-like element involved in anoxic specific inducibility								
	HSE	AAAAAATTTC	cis-acting element involved in heat stress responsiveness	2		2	2	1	1	2	1
	HSE	CNNGAANNTTC-NNG	cis-acting element involved in heat stress responsiveness								
	LTR	CCGAAA	cis-acting element involved in low-temperature responsiveness	1	2						
	TC-rich repeats	ATTTTCTTCA	cis-acting element involved in defense and stress responsiveness	3	3	1					1
	TC-rich repeats	GTTTTCTTAC	cis-acting element involved in defense and stress responsiveness	1					1		
	TC-rich repeats	ATTCTCTAAC	cis-acting element involved in defense and stress responsiveness					1			
	Box-W1	TTGACC	fungal elicitor responsive element			2			3	1	1
	AT-rich sequence	TAAAATACT	element for maximal elicitor-mediated activation (2copies)						1		
	CCAAT-box	CAACGG	MYBHv1 binding site		1	1					1
	MBS	CGGTCA	MYB Binding Site			1				1	
	MBS	TAACTG	MYB binding site involved in drought-inducibility								
	MBS	CAACTG	MYB binding site involved in drought-inducibility	1			2			1	
	WUN-motif	TCATTACGAA	wound-responsive element								
	WUN-motif	AAATTTCCT	wound-responsive element								
			TOTAL	10	8	7	5	2	7	8	6

	Site Name	*PGSC0003DMG400015602*	*PGSC0003DMG400024474*	*PGSC0003DMG400029338*	*PGSC0003DMG400007621*	*Snakin-3*	*PGSC0003DMG401019533*	*PGSC0003DMG400033044*	*PGSC0003DMG400001226*	*PGSC0003DMG400001227*	*PGSC0003DMG401001384*
HORMONES	AuxRR-core										
	TGA-box										
	TGA-element							1		1	
	CGTCA-motif			1			2		1		
	TGACG-motif			1			2		1		
	ERE	3	1	1			1			1	
	GARE-motif		1	1			1				
	GARE-motif			1	1		1				
	TATC-box			1							
	P-box		1			1		2			

Table 1 (continued)

	Site Name	*PGSC0003DMG400015602*	*PGSC0003DMG400024474*	*PGSC0003DMG400029338*	*PGSC0003DMG400007621*	*Snakin-3*	*PGSC0003DMG401019533*	*PGSC0003DMG400033044*	*PGSC0003DMG400001226*	*PGSC0003DMG400001227*	*PGSC0003DMG401001384*
	P-box										
	TCA-element			1		1					1
	TCA-element										1
	TCA-element		1	1							
	TCA-element										1
	ABRE										
	ABRE										
	ABRE										
		3	4	8	1	2	7	3	2	2	3
STRESS	ARE	1		1	3			3	2		
	GC-motif		1	1							
	HSE	1	1		1				1	1	2
	HSE				1						
	LTR		1								
	TC-rich repeats	2			1	2		1	1	1	4
	TC-rich repeats										
	TC-rich repeats									1	
	Box-W1		1		1			1	1		2
	AT-rich sequence		1	1							
	CCAAT-box							1			
	MBS							1			
	MBS							1	1		2
	MBS				1			1			
	WUN-motif							1			
	WUN-motif								1		
		4	5	3	8	2	0	10	7	3	10

that *Snakin-1* was downregulated and *Snakin-2* was unaltered while *Snakin-3* was induced 24 h after infection. In addition, *in vitro* grown plants were inoculated with *Rhizoctonia solani* and whole-plant samples were harvested at 7, 10 and 12 days after infection. The *Snakin-1* expression was slightly induced, while *Snakin-2* and *Snakin-3* were downregulated after inoculation with the fungus (Fig. 6).

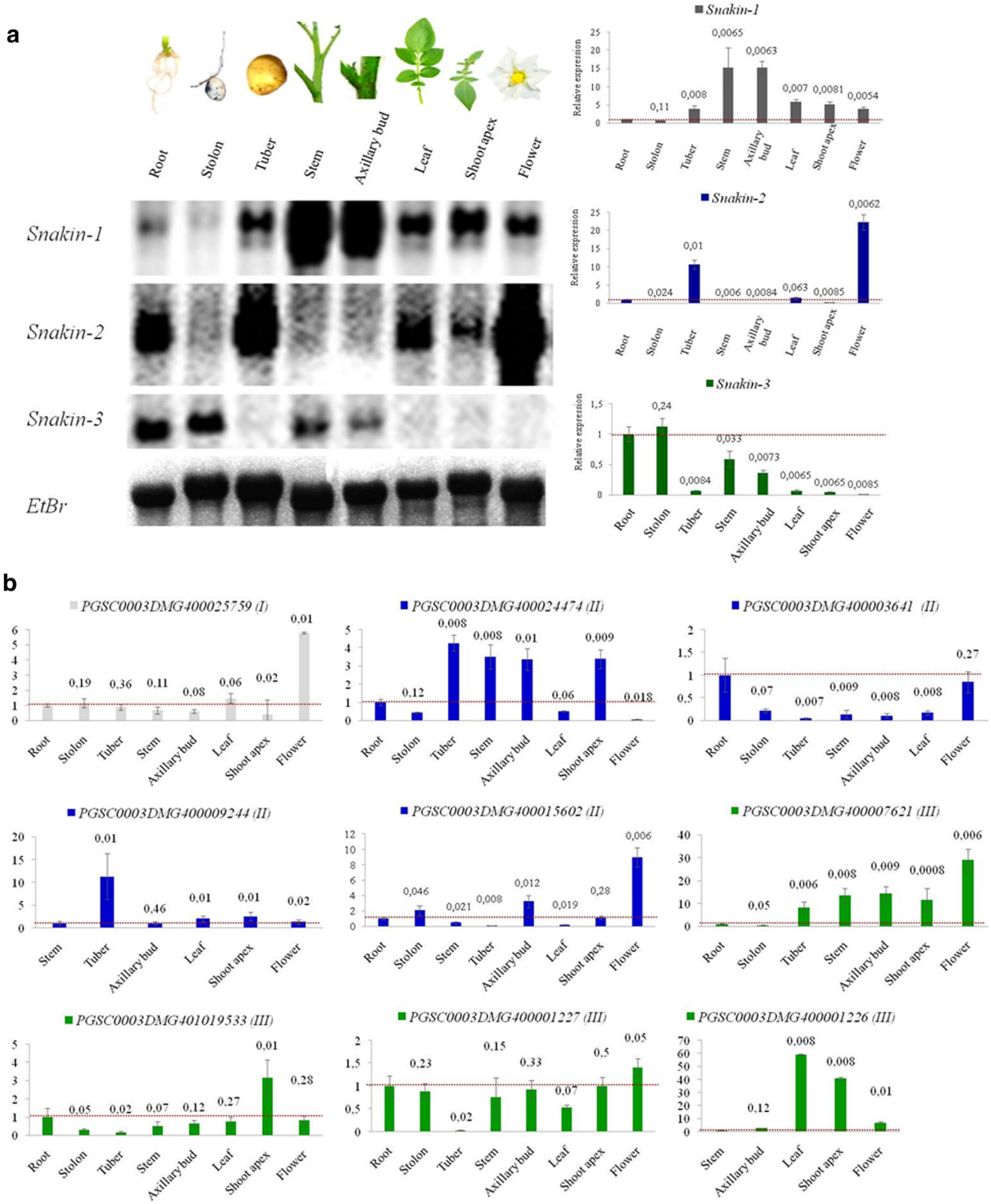
Discussion

In this study, we isolated, sequenced and characterized 16 Snakin/GASA genes from the potato cultivar Kennebec taking advantage of the recent availability of the potato genome sequence (Xu et al. 2011). This finding confirms that in potato this family consists of at least 18 members. In agreement, in a recent work on the structure and expression of *Snakin-1* and *Snakin-2*, Meiyalaghan et al. mentioned the occurrence of 18 GASA-like genes in potato (Meiyalaghan et al. 2014). We describe for the first time detailed information of these potato Snakin/GASA genes: genomic structures, phylogeny, conserved motifs/*cis*-elements in their promoter sequences and regulation of their expression.

All potato Snakin/GASA family members have structural characteristics typical of secreted polypeptide molecules. Each putative protein contains an N-terminal 18–29 amino acid hydrophobic region that that might act as a cleavable signal sequence. Even though the putative localization for some of Snakin/GASA peptides has been proposed based on sequence analyses, the subcellular localization of only a few of them have been experimentally determined. In this respect, the localization is variable among different family members. For example, GIP1 was found in the endoplasmic reticulum (Ben-Nissan et al. 2004), OsGASR peptides are restricted to the apoplasm or cell wall, while OsGSR1 protein localizes to the plasma membrane, cytoplasm and nucleus (Furukawa et al. 2006; Wang et al. 2009). In addition, GASA5 localizes to the cell wall and/or extracellular matrix, whereas a yellow fluorescent protein (YFP) fusion protein indicated that GsGASA1 is localized to the plasma membrane, cytoplasm and nucleus (Zhang et al. 2009; Li et al. 2011). More recently, Snakin-1 and GASA14 were found to localize to the plasma membrane (Nahirñak et al. 2012; Sun et al. 2013). The evidence reveals that despite the presence of a putative signal peptide and the lack of other predicted targeting signals, not all the Snakin/GASA proteins are targeted to the cell wall/extracellular matrix. Therefore, experimental evaluation of the in silico localization predictions is required. Many factors may be responsible for the localization of these proteins, such as post-translational modifications, electrostatic interactions, covalent bonds to membrane lipids or attachment/interaction with other proteins. Downstream the putative signal peptide, a variable region and a conserved C-terminal domain can be defined. Interestingly, all of them maintain 12 cysteines of the C-terminus in highly conserved positions of the aminoacid sequences that are essential for their biochemical activity and responsible for their protein structure. In this context, it has been speculated that these proteins may play a role in reactive oxygen species scavenging due to the presence of these redox-active cysteines (Wigoda et al. 2006; Rubinovich and Weiss 2010). Rubinovich and Weiss demonstrated the redox activity of *GASA4 in planta* and *in bacteria*, and they also showed that the replacement of four of the conserved cysteines in this region with alanine residues inhibited the antioxidant activity of *GASA4* (Rubinovich and Weiss 2010). Recently, they demonstrated that as *GASA4*, *GASA5* also acts as an antioxidant despite their opposite biological activities: promotion and inhibition of GA responses respectively (Zhang et al. 2009; Rubinovich and Weiss 2010; Rubinovich et al. 2014).

The Snakin/GASA genes were found to be distributed on nine of the 12 chromosomes in potato. In maize, *ZmGSL1* to *ZmGSL10* map to seven of the 10 chromosomes with chromosome 2 and chromosome 5 harboring three and two loci respectively. Similarly, the nine rice Snakin/GASA homologs are distributed over six of the 12 rice chromosomes with chromosomes 3, 5, and 6 accommodating two members (Zimmermann et al. 2010). Even though Snakin/GASA proteins have been classified as CRPs (Silverstein et al. 2007) they do not share their chromosomal distribution, since CRP-encoding genes are usually present in gene clusters located in discrete chromosomal regions (Marshall et al. 2011).

The evolutionary relationship within the Snakin/GASA family was examined by constructing a phylogenetic tree based on multiple sequence alignments of the full-length predicted proteins. The Snakin/GASA members in potato appear to form three phylogenetic groups. This is consistent with previous studies (Berrocal-Lobo et al. 2002) and with phylogenetic trees based on Snakin/GASA proteins of others species (Furukawa et al. 2006; Zhang and Wang 2008; Zimmermann et al. 2010). Phylogenetic diversity does not always reflect functional differences. For example, in strawberry, FaGAST1 and FaGAST2 showed differences in their expression pattern and were reported to belong to different subfamilies, however they were involved in similar physiological functions (Moyano-Canete et al. 2013). Likewise, Snakin-1 and Snakin-2 are grouped in different subfamilies and both were implicated in pathogen resistance (Segura et al. 1999; Berrocal-Lobo et al. 2002; Almasia et al. 2008; Faccio et al. 2011; Balaji and Smart 2012; Rong et al. 2013; Mohan et al. 2014). Moreover, belonging to the same subfamily does not imply having the same function. For example, GASA4 and GASA5 are grouped together and were shown to be involved in different roles (Zhang et al. 2009; Rubinovich and Weiss 2010). Therefore, predictions of possible functions of the recently identified potato Snakin/GASA


a
Root
Stolon
Tuber
Stem
Axillary bud
Leaf
Shoot apex
Flower
Snakin-1
Snakin-2
Snakin-3
EtBr
Relative expression
0,11
0,008
0,0065
0,0063
0,007
0,0081
0,0054
0,024
0,01
0,006
0,0084
0,063
0,0085
0,0062
0,24
0,033
0,0073
0,0065
0,0065
0,0085
b
PGSC0003DMG400025759 (I)
0,19
0,36
0,11
0,08
0,06
0,02
0,01
PGSC0003DMG400024474 (II)
0,12
0,008
0,008
0,01
0,06
0,009
0,018
PGSC0003DMG400003641 (II)
0,07
0,007
0,009
0,008
0,008
0,27
PGSC0003DMG400009244 (II)
0,46
0,01
0,01
0,02
0,01
PGSC0003DMG400015602 (II)
0,046
0,021
0,008
0,012
0,019
0,28
0,006
PGSC0003DMG400007621 (III)
0,05
0,006
0,008
0,009
0,0008
0,006
PGSC0003DMG401019533 (III)
0,05
0,02
0,07
0,12
0,27
0,01
0,28
PGSC0003DMG400001227 (III)
0,23
0,02
0,15
0,33
0,07
0,5
0,05
PGSC0003DMG400001226 (III)
0,12
0,008
0,008
0,01

◀ **Fig. 4** Expression of potato Snakin/GASA genes in different tissues. (a) *Northern blot* (left part) and qRT-PCR (right part) analysis of *Snakin-1*, *Snakin-2* and *Snakin-3*. (b) qRT-PCR analysis of the spatial expression of another member of each subfamily: *PGSC0003DMG400025759* (I), *PGSC0003DMG400024474* (II), *PGSC0003DMG400003641* (II), *PGSC0003DMG4000092444* (II), *PGSC0003DMG4000015602* (II), *PGSC0003DMG400007621* (III), *PGSC0003DMG401019533* (III), *PGSC0003DMG400001227* (III) and *PGSC0003DMG400001226* (III). I, II, III: Subfamily I, Subfamily II and Subfamily III. Red lines correspond to expression level in root and expression ratios in different tissue samples are calculated in relation to root

genes based on phylogenetic relationships and the available data in the literature may not be so reliable.

An analysis of the intron/exon structures of potato Snakin/GASA genes revealed that the coding sequences of all these genes are interrupted by introns and the number of exons varied from two to four. Furthermore, they displayed different structural pattern of exon–intron junctions even within the same phylogenetic subgroup.

Even though the first member of Snakin/GASA family was described in 1992, their functions are not completely elucidated and there is no apparent consensus in the roles they play. In this context, we performed studies on the putative promoter sequences and expression patterns to gain insight in this issue. Bioinformatic analysis revealed that potato Snakin/GASA genes upstream sequences carry a variety of potential *cis*-acting elements, including binding sites for transcription factors that are regulated by hormones and stress responsive elements. The patterns of *cis*-acting elements differ

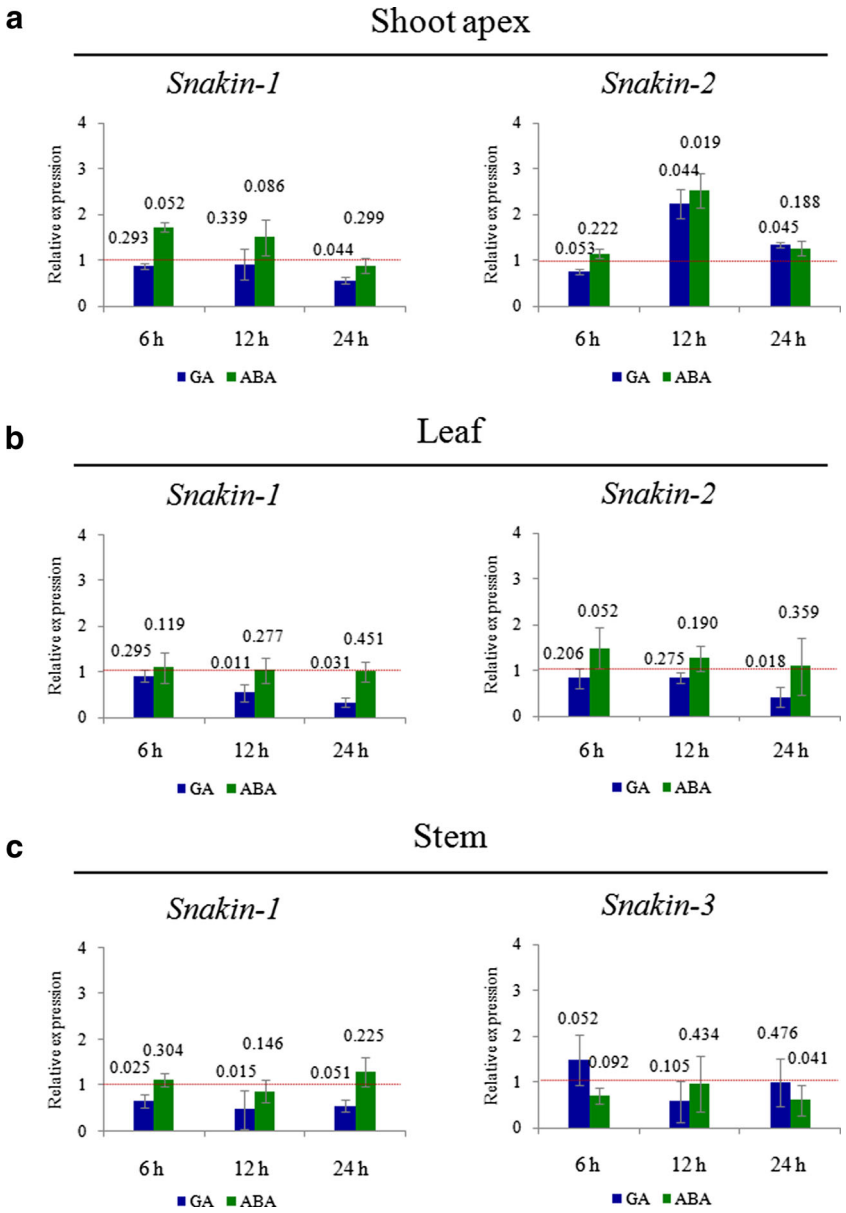


Fig. 5 Response of potato Snakin/GASA genes to GA or ABA treatment qRT-PCR analysis of total RNAs extracted from (a) shoot apex, (b) leaves and (c) stems of mock-treated plants (Control), or plants treated with 100 μM ABA or 100 μM GA_3. Red lines correspond to expression level of control plants in the corresponding time point

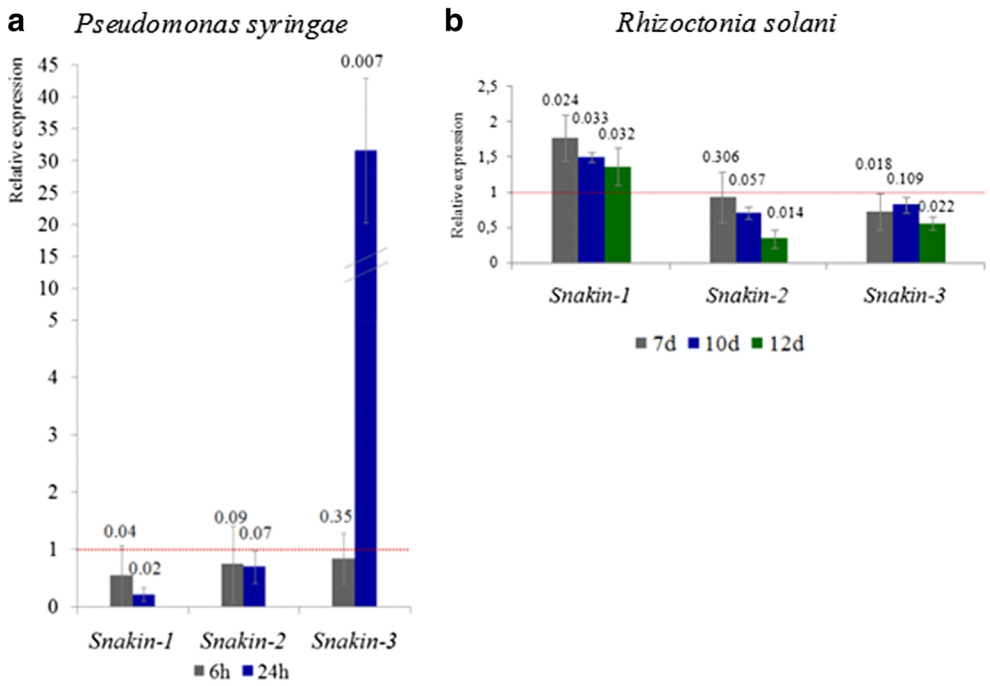


Fig. 6 Response of potato Snakin/GASA genes to pathogen infection qRT-PCR analysis of total RNA. (a) For P. *syringae* pv. *tabaci* infection, fully expanded leaves of 6-week-old potato plants were inoculated with bacteria or 10 mM $MgCl_2$ (control plants) and leaf samples were collected at 6 and 24 h after inoculation. (b) For *Rhizoctonia solani* infection, a PDA block with fungus mycelium was placed in the center of a magenta box carrying aseptically grown 4-week-old plants. Whole-plant samples were harvested at 7, 10 and 12 days after inoculation. Red lines correspond to expression level of control plants in the corresponding time point

among the potato Snakin/GASA members; which suggests that these genes may be differentially expressed in response to various stimuli. However, experimental validation is required to confirm the functionality of the predicted regulation sites.

In the present study, we found that spatial regulation of potato Snakin/GASA genes is highly specific. Moreover, no link was found between the expression patterns and the clustering obtained by phylogenetic analysis. Most of the expression results obtained in *Solanum tuberosum* cv Kennebec for *Snakin-1* and *Snakin-2* are in agreement with previously reported data (Segura et al. 1999; Berrocal-Lobo et al. 2002), although we found some differences. A possible explanation for the observed differences could be the distinct genotype analyzed or experimental variation (for example, plant age at the moment of sampling). This was already observed in previous studies in which *Snakin-1* spatial expression pattern in two potato varieties also revealed some differences (Segura et al. 1999; Berrocal-Lobo et al. 2002; Almasia et al. 2008). Moreover, RNA-seq data reported by the Potato Sequencing Consortium also showed variations from previous northern analysis and expression studies performed here which may reflect differences in cultivar and growth conditions (Segura et al. 1999; Berrocal-Lobo et al. 2002; Almasia et al. 2008; Xu et al. 2011; Meiyalaghan et al. 2014). Similar observations were described in another species: *GIP1* is differentially expressed in two distinct petunia varieties (Ben-Nissan et al. 2004).

Snakin/GASA genes are involved in multiple signaling pathways such as stress responses or developmental processes and most of them are regulated by plant hormones (Nahirnak et al. 2012). In this work, we found that *Snakin-1* expression is downregulated by GA and it seems not to be affected by ABA in shoot apex, leaf or stem. Segura et al. (Segura et al. 1999) reported no response of *Snakin-1* to a number of hormone treatments, including GA; in their study, however, they analyzed young potato leaves in which developmentally regulated expression of the *Snakin-1* gene had not been detected. The regulation of *Snakin*-2 appeared to be more complex. It was induced by GA 12 h and 24 h after treatment in shoot apex and it was repressed by GA 24 h after treatment in leaf. In addition, the *Snakin*-2 gene expression was induced by ABA 12 h after treatment in the shoot apex. Berrocal-Lobo et al. (2002) had shown that *Snakin-2* was highly induced by ABA in potato leaves and to a lesser extent in stem, while expression levels of *Snakin-2* were repressed by GA in both tissues. The observed differences in expression could be due to the experimental design or the distinct genotypes studied. Finally, *Snakin-3* expression was not affected by GA at least in the three tissues analyzed and in the experimental conditions tested and it was downregulated by ABA 24 h after treatment in stem. It is important to note that although we evaluated the expression of the 3 genes in 3 different tissues to have a more complete view about hormonal regulation, we did not detect *Snakin-2* expression in stem or *Snakin-3* in shoot apex or leaf; these results are in agreement with spatial expression analyses presented in Fig. 4, where we demonstrate that they are differentially expressed in different parts of the plant. In sum, our results indicate that Snakin-GASA gene regulation in potato depends not only on the hormone and the organ affected but also on the time elapsed after treatment. Similar complex regulation by hormones has been reported previously. For example, in Arabidopsis the regulation of *GASA4* expression by

GA is tissue specific: it is upregulated in meristematic regions and repressed in cotyledons and leaves (Aubert et al. 1998). Moreover, Li et al. (Li et al. 2011) have recently demonstrated that, under the influence of GA, the expression pattern of *GsGASA1* in leaves and roots is more complex, since it depends not only on the organ affected but also on the treatment length. Even though we observed changes in gene expression after hormone treatment in our conditions, it would be interesting to evaluate regulation of Snakin/GASA genes in earlier time points; since if they are early responsive genes more drastic expression changes might be observed. Moreover, it would be very interesting to correlate the transcript regulation with protein levels, however, the unavailability of specific antibodies makes this issue still intriguing. Finally, we also investigated the effect of pathogen infection on the expression of these genes and we observed that *Snakin-1*, *Snakin-2* and *Snakin-3* expression is affected by bacterial and/or fungal inoculation. These results are consistent with the participation of *Snakin-1* and *Snakin-2* genes in biotic stress tolerance. Moreover, *Snakin-3* pathogen-responsive expression patterns are congruent with a putative defense role.

Conclusions

Snakin/GASA genes have been involved in different aspects of plant growth and development, however, the exact role played by these proteins is still intriguing. The identification of a large number of these genes in distantly related species highlights the importance of these proteins and suggests that they fulfill essential functions in plants. In this study, Snakin/GASA genes from the commercially cultivated *S. tuberosum subsp. Tuberosum* cv. Kennebec genotype have been characterized. Putative potato Snakin/GASA proteins can be grouped in 3 subfamilies but predictions of possible functions based on phylogenetic relationships and the available data in the literature may not be so reliable. We found that spatial and hormonal regulation of potato Snakin/GASA genes is highly specific and dependent on the plant developmental stage, the organ affected by the hormone and time elapsed after treatment. Moreover, comparisons of our results and data previously published, revealed that expression pattern of Snakin/GASA genes is also dependent on the genotype analyzed. Interestingly, we found that in this cultivar *Snakin-1*, *Snakin-2* and also *Snakin-3* expression is affected by bacterial and/or fungal inoculation. These results strengthen the participation of *Snakin-1* and *Snakin-2* genes in biotic stress tolerance and suggest that *Snakin-3* is also involved in biotic stress tolerance. In sum, the data presented here could be a good starting point for more focused and deep investigations regarding the biological functions of potato Snakin/GASA genes during plant development and in response to environmental stress.

Acknowledgments This research was supported by PEAEBIO 245001/711, PNBIO 1131022/3 (INTA), AECID D/024562/09 and PICT 2011-1377. We thank Julia Sabio for her invaluable help as English editor. Sequencing service and bioinformatics analyses were performed at National Institute of Agricultural Technology, Biotechnology Institute, Genomics Unit and Bioinformatics Unit respectively.

Author Contribution Statement VN participated in the design of the study, carried out the molecular techniques and drafted the manuscript. MGU contributed to data interpretation and helped to draft the manuscript. MR participated in the design of the study and contributed to data interpretation. NP helped to draft the manuscript. HEH assisted in the interpretation of the results and contribute to writing the manuscript revising it critically. NIA contributed to data interpretation and helped to draft the manuscript. CVR conceived and coordinated the study, contributed to the work by the discussion of the data and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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