Journal of Biotechnology 283 (2018) 62–69

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Potato *snakin-1* gene enhances tolerance to *Rhizoctonia solani* and *Sclerotinia sclerotiorum* in transgenic lettuce plants

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ARTICLE INFO

Keywords:
Lettuce
Phytopathogens
Snakin-1
Antimicrobial peptide
Transgenesis
Challenge trials

ABSTRACT

Snakin-1 is a cysteine-rich antimicrobial peptide (AMP) isolated from potato tubers, with broad-spectrum activity. It belongs to the Snakin/GASA family, whose members have been studied because of their diverse roles in important plant processes, including defense. To analyze if this defensive function may lead to disease tolerance in lettuce, one of the most worldwide consumed leafy vegetable, we characterized three homozygous transgenic lines overexpressing Snakin-1. They were biologically assessed by the inoculation with the fungal pathogens *Rhizoctonia solani* and *Sclerotinia sclerotiorum* both *in vitro* and *in planta* at the greenhouse. When *in vitro* assays were performed with *R. solani* on Petri dishes containing crude plant extracts it was confirmed that the expressed Snakin-1 protein has antimicrobial activity. Furthermore, transgenic lines showed a better response than wild type in *in vivo* challenges against *R. solani* both in chamber and in greenhouse. In addition, two of these lines showed significant *in vivo* protection against the pathogen *S. sclerotiorum* in challenge assays on adult plants.

Our results show that Snakin-1 is an interesting candidate gene for the selection/breeding of lettuce plants with increased fungal tolerance.

1. Introduction

Lettuce (*Lactuca sativa* L.) is an annual plant of the *Asteraceae* family. Various types of lettuce are cultivated across the globe, especially in temperate and subtropical regions. It plays an important role in diet and nutrition as the world's most consumed leafy salad vegetable (Mou, 2012). Lettuce is highly perishable and cannot be stored or transported during long periods of time. Thus, large volumes of lettuce are cultured three or four times a year in the same field/greenhouse increasing the possibility of suffering pests and diseases, which are controlled by genetic resistance, cultural practices and mostly by agrochemical protection. The use of pesticides not only increases production costs but it is also unsafe to the environment, farmers and even consumers if they are used inappropriately. This is a really important issue as lettuce is one of the most cultivated vegetables by small-scale farmers in the suburbs of metropolitan areas.

When genetic sources of disease resistance are available in wild relatives, breeding is the cleanest and the most cost-effective method to obtain resistant plants. Nevertheless, this improvement is not always possible and this situation can be overcome by transgenesis. A stable transformation system in lettuce has enabled the introduction of several genes oriented to molecular breeding. Thus, transgenic lettuce lines with resistance to virus (Dinant et al., 1997; Kawazu et al., 2016) and fungal pathogens (Dias et al., 2006; Govindarajulu et al., 2015; Okubara et al., 1997) were obtained.

Fungi and viruses are important infectious diseases in lettuce (Raid, 2004). In the case of fungal pathogens, *Rhizoctonia solani*, *Sclerotinia* spp., *Pythium* spp. and *Botrytis cinerea* are among the main causes of basal rot in undercover cultivation, especially in winter (Van Beneden et al., 2009; Wareing et al., 1986).

R. solani is a soil-borne fungal pathogen causative of bottom rot that infects all types of lettuce and may be found wherever this crop is grown (Raid, 2004). Favored by warm and wet conditions, the pathogen is capable of direct penetration, entering the plant through healthy or wounded tissues (Herr, 1993) and can cause losses as high as 70%.

Drop is one of the most widespread and destructive diseases worldwide in lettuce production (Purdy, 1979; Subbarao, 1998). It is

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https://doi.org/10.1016/j.jbiotec.2018.07.017
Received 5 April 2018; Received in revised form 10 July 2018; Accepted 11 July 2018

caused by *Sclerotinia minor* Jagger and *S. sclerotiorum* (Lib.) de Bary, being the last one the most prevalent species in Argentina (Granval de Millán and Gaviola, 1991). *S. sclerotiorum* infects lettuce plants by airborne ascospores (Abawi and Grogan, 1979; Adams and Ayers, 1979) and more infrequently by direct germination of sclerotia. Leaves usually have water-soaked lesions that develop into necrotic tissues that subsequently develop patches of fluffy white mycelium, often with sclerotia (Bolton et al., 2006).

To counteract the attack of pathogens, plants have evolved several defense mechanisms, including the synthesis of antimicrobial peptides (AMPs). AMPs are part of innate immunity, establishing a first line of defense against pathogens. Most of them are cysteine (Cys)-rich peptides expressed in nearly all organs constitutively or in response to infections, being a component of the barrier defense system. They are classified according to their structure and the number of Cys present on it, in different families such as cyclotides, defensins, thionines, LTP proteins, hevein-like, vicilin-like and knottin-like peptides and snakins (Stotz et al., 2013).

Snakin-1 and Snakin-2 were the first snakins identified, both of them were isolated from potato (*Solanum tuberosum*) tubers and they were shown to exhibit antimicrobial activity *in vitro* (Berrocal-Lobo et al., 2002; Segura et al., 1999). Interestingly, overexpression of the *snakin-1* gene in potato plants enhances *in vivo* resistance to *R. solani* and *Erwinia carotovora* (Almasia et al., 2008). Moreover, heterologous transgenic expression of Snakin-1 in wheat has demonstrated *in vivo* antimicrobial activity against *Blumeria gramimis* and *Gaeumannomyces graminis* (Faccio et al., 2011; Rong et al., 2013).

They belong to the Snakin/GASA family, because of their similarity with members of the GAST family from tomato (Gibberellic Acid Stimulated Transcript) and the GASA family from *Arabidopsis* (Gibberellic Acid Stimulated in *Arabidopsis*). Snakin/GASA proteins are involved in a diverse range of functions including hormonal crosstalk, development, and defense against biotic and abiotic stress (Nahirñak et al., 2012a,b). They have a C-terminal region of approximately 60 aminoacids with 12 Cys residues in conserved positions, named GASA domain (Aubert et al., 1998), responsible for the protein structure which may be essential for their biochemical activity as antioxidants. It has been speculated that these proteins may play a role in reactive oxygen species scavenging due to the presence of these redox-active Cys (Rubinovich and Weiss, 2010; Wigoda et al., 2006).

In this work we assessed the direct antifungal activity of transgenic plant extracts against *R. solani* and evaluated the antimicrobial activity of Snakin-1 in the defense against necrotrophic fungi *in planta*. The results showed that Snakin-1 overexpressing transgenic plants have a better response with respect to non-transgenic plants when challenged with *R. solani* or *S. sclerotiorum*.

2. Material and methods

2.1. Biological materials and growth conditions

2.1.1. Plant material

Plants corresponding to the T4 generation from three independent transgenic lettuce lines carrying *snakin-1* sequence from *Solanum chacoense* between the *rbcS1* promoter and the *T35S* terminator and a kanamycin resistance cassette (*nopaline synthase* promoter *Pnos*, *nptII* gene and *Tnos* terminator) and one non-transgenic (NT) control line were used. These homozygous transgenic lines were obtained as described in Darqui et al. (2017) where Southern blot analysis showed that 1.7 A and 2.2B lines carried multiple insertion events, with at least four and two integrations respectively, while 4.2.2C line had a single insertion event. In the present work, 1.7 A, 2.2B and 4.2.2C transgenic lines were analyzed (for practical purposes they were renamed as L1, L2 and L3, respectively). All lines were obtained transforming the Grand Rapids cultivar; a loose leaf type lettuce originated in Michigan (USA) and released prior 1890 (Mikel, 2007), which has not known resistance nor tolerance to the fungal pathogens tested in this work.

In all tests described herein, transgenic and NT samples were collected from plants of the same age and physiological stage, harvested at the same time and kept under the same storage conditions.

2.1.2. Fungal strains

Rhizoctonia solani AG-3 strain and *Sclerotinia sclerotiorum* were kindly provided by the Área de Bioinsumos Microbianos, IMyZA (INTA, Argentina) and EEA Balcarce (INTA, Argentina), respectively. They were cultivated and maintained on potato dextrose agar medium (Difco™ PDA, BD company, USA) in a laboratory thermostat incubator (MiLab, China) at 28 °C and 25 °C, respectively, with transfer of an agar disc to fresh medium every 30 d.

2.2. Molecular analysis

2.2.1. RNA extraction and cDNA synthesis

Leaf tissue samples were obtained from rosette growth stage plants (16 day old), cultivated at greenhouse in 350 mL pots. Material extraction was done from five plants, one sample per plant, for each line (transgenic and NT). Samples were frozen in liquid nitrogen and stored at −80 °C till RNA extraction. Total RNA was isolated using Trizol (Invitrogen, USA) extraction system according to the manufacturer's instructions and quality was evaluated by spectrometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). Total RNA (1 μg) was digested with 1 U DNase I and DNase 1X reaction buffer (Invitrogen, USA) before cDNA synthesis. DNase-treated RNA was checked through PCR to verify the absence of DNA contamination. The cDNAs were synthesized using Superscript III (Invitrogen, USA) and oligo-dT primers according to manufacturer's instructions.

2.2.2. Design and evaluation of oligonucleotide primers for qPCR

Actin (*ACT*) and *Elongation Factor 1 alpha* (*EF1α*) were used as reference genes. The lettuce *ACT* mRNA sequence was extracted from GenBank database. The *EF1α* mRNA sequence was identified by screening the lettuce EST sequences from GenBank database, using the *Helianthus annuus* homologue as the query. The *snakin-1* mRNA (*SN1*) sequence from *S. chacoense* was obtained from GenBank database.

Primer pairs were designed using Primer Express software (Applied Biosystems, USA) and their specificities were tested by sequencing the obtained PCR products (Table 1).

2.2.3. Quantitative polymerase chain reaction (qPCR)

Reactions were performed in 96-well plates in a StepOnePlus cycler

Table 1
Reference genes, primers and parameters derived from q-PCR analysis.

Gene name	GenBank accession number	Primer sequences (forward/reverse)	Tm (°C)	Amplicon length (bp)
ACT	**AY260165.1**	CAACTGGGATGACATGGAAAAGA	61	100
	(L. sativa)	GAGAGGTGCCTCAGTGAGAAGAAC	66.3	
EF1α	AY094064.1	CGAGGAAATCGTGAAGGAAGTG	62.7	100
	(H. annuus)	GTTGTCACCTTCGAATCCAGAGA	62.8	
SN1	**EF206290.1**	TCACTCTTGTTATTACCCCTTCTCTCA	63.1	100
	(S. chacoense)	GCAAGTCCTGCCTTTGAACATC	62.7	

(Applied Biosystems, USA). Each reaction was carried out in duplicate and contained 10-fold diluted cDNA template, 300 nM of each primer, and 1X FastStart Universal SYBR Green Master (Roche, Switzerland), in a final volume of 13 µL. Two non-template controls were included in each run for each set of primers. The thermal profile was 95 °C for 10 min activation and denaturation, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The threshold cycle numbers (Cq) were automatically determined by the StepOnePlus software. Primer efficiencies were calculated using the LinRegPCR software (Ruijter et al., 2009). *Snakin-1* transcription levels were calculated according to the transcription levels of *ACT* and *EF1α* reference genes. Relative expression ratios and statistical analysis were performed using the fgStatistics software (Di Rienzo, 2012).

2.2.4. Immunoblot analysis

Leaf tissues from NT, L1, L2 and L3 plants were ground in mortar with liquid nitrogen. Next, 250 mg of each sample plus 250 µL of extraction buffer (10 mM KCl, 5 mM $MgCl_2$, 400 mM sucrose, 100 mM Tris-HCl (pH 8), 10% glycerol and 1 mM PMSF) were incubated at room temperature for 5 min and then centrifuged at 5000 rpm for 3 min. The supernatant (200 µL) was transferred to a new tube and concentrated in a Concentrator Plus equipment (Eppendorf, Germany). Total proteins were quantified by the Bradford (1976) technique. From each sample, 300 µg of total proteins was taken and brought to a final volume of 150 µL with extraction buffer and 50 µL of 4X loading buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.008% bromophenol blue, 0.25 M Tris-HCl pH 6.8) was added. Samples were heated to 100 °C for 5 min and stored at −20 °C until the moment of use.

A 15% separation gel was prepared (0.375 M Tris HCl buffer pH 8.8, 0.1% SDS, 120 g/L acrylamide, 3.2 g/L bisacrylamide, 0.05% ammonium persulfate (APS) and 0.5 µL/mL TEMED). The stacking gel contained 0.1 M Tris HCl buffer pH 6.8, 0.083% SDS, 33 g/L acrylamide, 0.88 g/L bisacrylamide, 0.04% APS and 0.83 µL/ML TEMED.

The electrophoretic run was performed in 1X running buffer (25 mM Tris-HCl, 200 mM glycine, 0.1% SDS) at 120 V and transferred to a nitrocellulose membrane at 4 °C for 90 min at 200 mA (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad).

Blocking, addition of antibodies and washing steps were performed at room temperature with gentle agitation and a milk TBS solution (10% w/v nonfat dried milk in Tris Buffer Saline) was used. The membrane was blocked for 1 h and incubated in a 1:1000 dilution of the specific antibody (Almasia et al., 2017) for 2 h, followed by three 5–10 min washes. Finally, it was incubated with the conjugated antibody (anti-mouse antibody, phosphatase-conjugated) in a 1:5000 dilution for 2 h and washed three times (5–10 min). For visualization the membrane was immersed in NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) dye solution for 10 min, and it remained in darkness until colour was observed. The reaction was stopped with the addition of water.

2.2.5. ROS detection assays

Superoxide anion (O_2^-) detection was conducted using an NBT staining method (Wohlgemuth et al., 2002). Hydrogen peroxide (H_2O_2) accumulation was analyzed using 3,3′-diaminobenzidine (DAB) (Thordal-Christensen et al., 1997). The same fifteen healthy greenhouse plants per line were used in both analyses in order to reduce variability between individuals. These tests were performed with a difference of 2 weeks to allow plant recovery after leaf discs detachment, while plants were still at the same physiological state of young rosette. The analyses were performed on wounded tissue, as 20 mm diameter discs cut form leaves were immediately submerged in the corresponding reaction buffer.

NBT staining: discs from the second leaf of 16 day old plants were taken and submerged in an NBT 0.1% solution in 50 mM potassium phosphate buffer (pH 7.8). DAB staining: similar samples were taken from the fifth leaf of 30 day old plants and submerged in a DAB-HCl 0.1% solution (pH 3.8). In each assay, both solutions were vacuum infiltrated into tissues for 2 min. The infiltrated leaf discs were incubated 2 h at room temperature, and later cleared in boiling ethanol 96% to remove pigments. Cleared leaf discs were scanned at 600 dots per inch (*ImageScanner*, Amersham Biosciences, UK). NBT reacts forming a blue formazan compound and DAB produces a red-brown precipitate. Leaf discs obtained from other plants of the same age (6 discs from 16 day old plants for the NBT test and 4 discs from 30 day old plants for the DAB test) were used as pigment-clearance controls. Staining intensities were quantified using ImageJ software (Schneider et al., 2012). A Student's *t*-test for independent samples (significance level α: 0.05; left tail) implemented in Infostat (Di Rienzo et al., 2016) was used to search significant differences among each transgenic line and the NT line in both treatments.

2.3. Pathogen infection assays

2.3.1. Effect of leaf extract from transgenic plants on R. solani mycelial growth in vitro

Three plants of NT and L1 transgenic line (with 3 technical replicates per plant) were used. Seven grams of ground tissue from 4-week-old plants were incubated with 20 mL of sodium phosphate buffer (pH 7.0) at room temperature during 30 min. The leaf extract was centrifuged twice at 12,000 *g* to remove cellular debris. The supernatant was filter-sterilized (syringe filter 0.22 µm, Merck Millipore, USA) and total protein content was quantified using the Bradford method. Sterile Petri dishes (90 × 15 mm) were dispensed with 20 mL of PDA medium plus 5 mg of sample total proteins. A 4-mm-diameter 5-d-old mycelial plug from *R. solani* grown in PDA was placed in the center of each plate and incubated at 28 °C.

Radial mycelial growth was recorded day by day until mycelium covered the entire plate surface. Plates were photographed and mycelial growth area (cm^2) was calculated using ImageJ at 1, 2, 3 and 4 d post inoculation (dpi). Statistical analyzes were done using Infostat. To detect significant differences a Tukey's test (α: 0.05) was performed. The dependent variable was "mycelial growth area" and the variable of classification was the "line" (line L1 or the NT line).

2.3.2. Infection assays on lettuce seedlings with R. solani

Challenge tests were performed as described by Beracochea et al. (2015) with modifications. T4 seeds from L1, L2, L3 and NT lines were sterilized as described by Radonic (2010), sown in 60 mL of 1/2 MS dispensed in sterile Petri dishes (145 × 20 mm) and kept in a growth chamber (23 °C, photoperiod: 16 h/8 h light/darkness). When the first true leaf arose in all seedlings, they were transferred to new medium agar plates (40 seedlings/plate). The plate area was divided in quarters. Two quarters were for NT seedlings while the remaining two quarters were for one of the transgenic lines. Three technical replicates per transgenic line were prepared. The seedlings were arranged in concentric discs. A 4-mm-diameter 5-d-old mycelial plug from *R. solani* grown in PDA was placed in the center of each plate. The development of the infection was monitored every 24 h until 7 dpi, when the entire plate was covered with mycelium and seedlings were classified as undamaged; moderately damaged (with at least one leaf affected by the fungus) and completely damaged/dead plant. For each line, contingency tables were constructed to compare the frequency of transgenic *versus* NT lettuce seedlings in the different classification categories. Statistical analyses were performed with a Pearson's χ^2 test ($p < 0.05$) in Infostat.

2.3.3. Infection assays on adult lettuce plants with R. solani

Thirteen to sixteen plants of transgenic and NT lines were germinated in 350 mL pots in a greenhouse and inoculated with *R. solani* at the rosette growth stage. Inoculation was performed in 5 leaves per plant in the adaxial surface by fixing one fungal disc per leaf with a transparent adhesive tape. Fungal discs were taken from the edge of

PDA plates, where mycelial growth was homogenous.

At the same time, 5 control plants from transgenic and NT lines were mock-inoculated with a free-fungal mycelium agar PDA disc. Inoculated plants were intercropped in plastic water-containing vessels located inside a transparent nylon cabin, in order to maintain high humidity conditions which facilitate fungus infection. Control plants were placed within a similar cabin. Plants were evaluated at 14 dpi and according to the severity of symptoms they were classified as dead, necrotic or slightly necrotic when infected tissue was restricted to the site of inoculation.

Each transgenic line was compared against the NT line through a Pearson's χ^2 test in contingency tables. Also, lines were compared considering the lesion area (cm^2) of leaves (slightly necrotic leaves were not included). Leaves were photographed in scanner. The lesion area was measured in each leaf using ImageJ. For dead leaves, the registered lesion area was established as 37 cm^2, since the most affected living necrotic leaf had a lesion area of 36.7 cm^2. A Student's *t*-test for independent samples (α: 0.05; left tail) was used to search significant differences among each transgenic line and the NT line.

2.3.4. Infection assays on adult lettuce plants with S. sclerotiorum

Seeds from transgenic and NT lines were germinated and grown in greenhouse, in 350 mL pots. Sixteen to twenty plants of each line, in the rosette growth stage, were inoculated by spraying 1×10^6 ascospores/mL on green tissues, according to the concentration used by Whipps et al. (Whipps et al., 2002). Since the fungi needs a nutrient source and free moisture to invade healthy leaf tissue, spores were diluted in the solution described by Leone and Tonneijck (1990): 62.5 mM KH_2PO_4 + 5.5 mM glucose.

At the same time, as control treatment, transgenic and NT plants were mock-inoculated with a nutrient solution without ascospores. Inoculated and mock-inoculated plants were placed separately inside two transparent nylon cabinets. All plants were wrapped in transparent nylon bags for the first 78 h, to ensure a high relative humidity, after that and until the end of the assay they were watered on the soil surface and water-sprayed on leaves surfaces.

Plants were evaluated at 8 dpi according to the presence of symptoms on infected leaves. Leaves were classified as unaffected (completely healthy green leaves) or affected (chlorotic, necrotic or dead leaves). In the case of a dead plant, all of their leaves were considered as dead. Each transgenic line was compared with the NT line in the number of affected leaves over the number of total leaves on each plant, through a Student's *t*-test for independent samples (α: 0.05; left tail). Since the variances were not homogeneous, the Satterthwaite correction was used (α: 0.05). Within the following days (up to 25 dpi) the occurrence of dead plants in each line was registered.

3. Results

3.1. Quantitative analysis of snakin-1 mRNA transcription in leaf tissue

Snakin-1 transcription levels for each transgenic line were quantified by qRT-PCR technique. Endogenous genes *EF1α* and *ACT* were used as internal controls (Fig. 1) being the average qRT-PCR efficiency 1.86 for the amplification of *EF1α*, 1.87 for the amplification of *ACT* and 1.87 for the amplification of *snakin-1*.

As the NT did not express the *snakin-1* transgene, values were relativized to the transcription levels of the line with the lowest *snakin-1* expression (L3). As shown in Fig. 1, L1 and L2 lines exhibited a higher relative expression of the *snakin-1* gene than L3 line. Particularly, L1 expression levels were approximately three more times than L2.

3.2. Analysis of Snakin-1 expression in leaf tissues

In order to study whether the heterologous protein Snakin-1 was translated in all transgenic lines, we performed Western blot analyses employing total protein extracts from transgenic and control plants. Extracts from transgenic plants exhibited a band with the expected electrophoretic mobility for heterologous Snakin-1 (6922 Da), which was absent in extracts of NT plants (Fig. 2). Anti-rSN1 serum successfully detected a more intense band in L1 and L2 lines than in L3 (Fig. 2), in concordance with qRT-PCR results (Fig. 1).

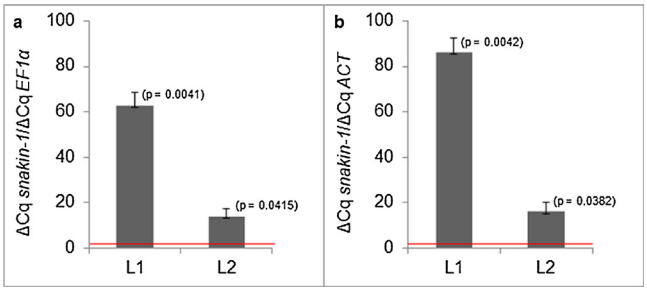

Fig. 1. Comparison of relative gene expression. Comparison of the relative expression of the *snakin-1* gene against (a) the *EF1α* endogenous gene and (b) the *ACT* endogenous gene. Ratio: ΔCq Target (Control-Sample)/ΔCq Reference (Control-Sample). Target: *snakin-1*. Reference: *EF1α/ACT*. Control: L3 (the line with the lowest Cq for the *snakin-1*gene, its expression level is indicated in red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Analysis of endogenous reactive oxygen species levels

Endogenous levels of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) were analyzed in transgenic and NT plants in order to study if Snakin-1 overexpression affects the redox balance. Images obtained after NBT and DAB staining were analyzed with ImageJ. They revealed reduced levels of ROS in two overexpressing lines. As shown in Fig. 3a, L2 (p-value = 0.0108) and L3 (p-value = 0.0037) exhibited lower levels of O_2^- when compared to the NT line. In the H_2O_2 evaluation, L2 (p-value < 0.0001) and L3 (p-value = 0.0002) exhibited lower levels of H_2O_2 than the NT line (Fig. 3b). However, there was no relation between *snakin-1* expression and ROS accumulation, since the line with the highest Snakin-1 expression (L1) had no differences with the NT line in terms of O_2^- / H_2O_2 accumulation.

3.4. Mycelial growth inhibition of the phytopathogenic fungi R. solani by extracts of lettuce plants overexpressing snakin-1

To assess the direct action of Snakin-1 against *R. solani* growth inhibition, the effect of transgenic plant crude extracts on mycelial radial growth was analyzed *in vitro*. Since L1 had shown the highest *snakin-1* levels (Fig. 1) we selected this line for this study. Fig. 4a shows representative inoculated plates containing crude leaf extract from a NT or transgenic plants. Mycelial growth was significantly inhibited along the entire bioassay (1, 2, 3 and 4 dpi) by the addition of extracts of L1 in the medium (Fig. 4b).

3.5. Infection assays of seedlings expressing Snakin-1 challenged against R. solani

To evaluate whether constitutive expression of Snakin-1 could play a role *in vivo* in pathogen protection, we assessed infection assays of seedlings. For this, seeds from L1, L2, L3, and NT lines were sown in sterile Petri dishes in quarter distribution and challenged with *R. solani.* All transgenic lines showed a higher percentage of undamaged seedlings compared to the NT line and this difference was statistically significant for L2 line (Fig. 5a). Fig. 5b shows a representative plate.

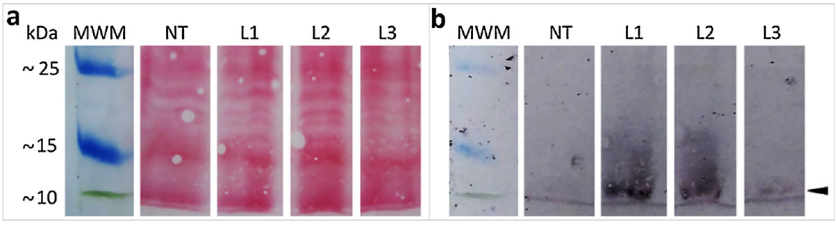

Fig. 2. Snakin-1 detected by Western blot analysis. (a) Membrane stained with Ponceau S Staining. (b) Immunoblot: Snakin-1levels detected in transgenic lines (L1-L2-L3). MWM: Molecular weight marker (Thermo Fisher Scientific, USA); NT: non-transgenic lettuce sample.

3.6. Challenge of adult lettuce plants expressing Snakin-1 against R. solani

To assess the effect of Snakin-1 in pathogenic adult plant–fungus interactions, we infected transgenic plants with *R. solani*. For this purpose, leaves were inoculated with PDA agar plugs containing mycelium and after 14 dpi plants were evaluated according to the type and area of lesion (Fig. 6a). Plants expressing Snakin-1 showed a tolerance response to *R. solani* infection since the ratio of dead leaves was lower for all transgenic lines when compared with the NT. Besides, the difference in the number of slightly necrotic, necrotic and dead leaves was statistically significant for lines L1 (p-value = 0.0924) and L2 (p-value = 0.0637) although not significant for line L3 (Fig. 6b).

Particularly, necrotic wound area in necrotic and dead leaves was measured and average lesion sizes were calculated for control and transgenic lines (Fig. 6c). All transgenic lines showed an increased tolerance to infection with *R. solani*, since the average lesion area was 13.71%, 10.28% and 11.17% for transgenic L1, L2 and L3 respectively while for NT it was 23% These differences was statistically significant for L1 (p-value = 0.045), L2 (p-value = 0.0058) and L3 (p-value = 0.0056) (Fig. 6c). These results demonstrated that Snakin-1 expression in adult lettuce plants confers significant protection against *R. solani*.

3.7. Challenge of adult lettuce plants expressing Snakin-1 against S. sclerotiorum

To assess whether the resistance displayed by the Snakin-1-over-expressing lines is extended to another fungal pathogen, transgenic and NT plants were challenged against the devastating necrotrophic pathogen *S. sclerotiorum*. Symptoms (3–5 mm-diameter necrotic lesions) were observed in all inoculated plants at 3 dpi, indicating the effectiveness of the inoculation method.

Several symptoms of varying degrees of severity, and a combination of them, were observed in leaves from inoculated plants at 8 dpi (Fig. 7a). The average number of affected leaves/total leaves was lower for all transgenic lines compared to the NT one (Fig. 7b), being statistically significant for lines L1 (p-value = 0.047) and L2 (p-value = 0.040).

At 11 dpi, transgenic lines had also a lower percentage of dead plants compared to the NT line and this behavior continued throughout the entire bioassay for L1 and L2 and until 17 dpi for L3. At the end of the evaluation (25 dpi) L2 showed a lower number (5.26%) of dead plants than the NT line (from 17.65%) (Fig. 8). These results indicate that progression of disease was slower in transgenic plants suggesting that Snakin-1 expression in adult lettuce plants confers tolerance to *S. sclerotiorum*.

4. Discussion

In the present work, we studied the effect of potato *snakin-1* gene expression in transgenic lettuce lines and its potential use for the breeding of plants with resistance to a broad spectrum of phytopathogens. As explained in the introduction section, plants have evolved a variety of non-specific defense mechanisms including the AMPs, which provide innate immunity by acting directly against a wide range of

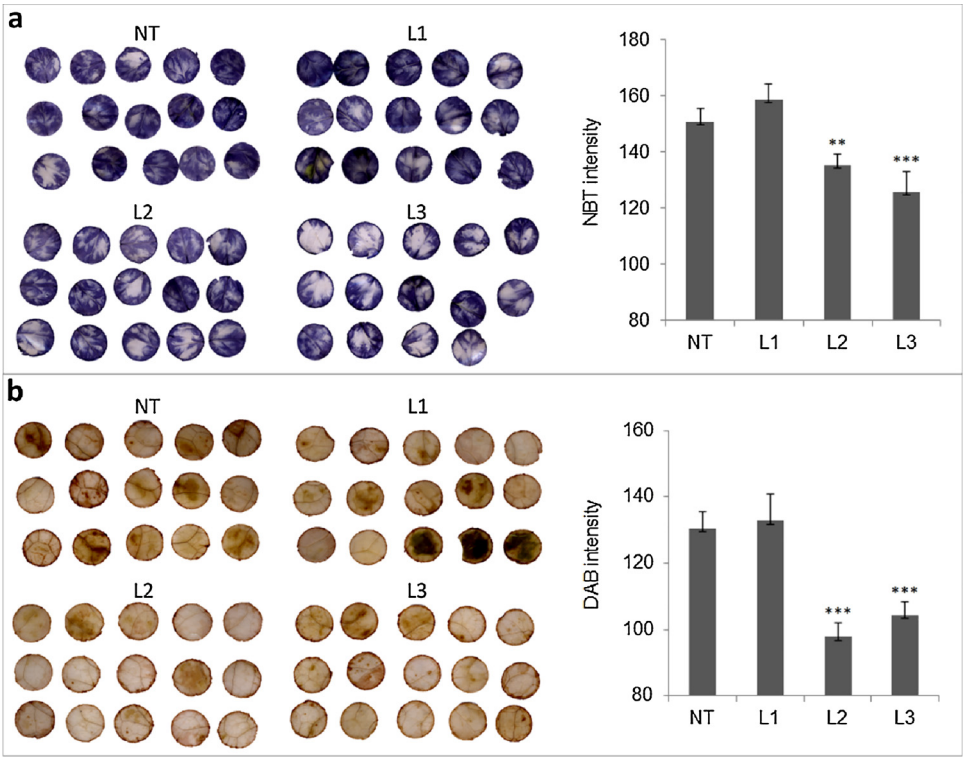

Fig. 3. ROS detection in Snakin-1 over-expressing transgenic lettuce lines. (a) O_2^- levels detected by NBT staining of leaf discs taken from transgenic and NT plants. Left: leaf discs after NBT staining. Right: average NBT intensities in each line analyzed by a t-test (α: 0.05; left tail) (***p-value < 0.01; **p-value < 0.05). (b) H_2O_2 levels determined by DAB staining of leaf discs taken from transgenic and NT plants. Left: leaf discs after DAB staining. Right: average DAB intensities in each line analyzed by a t-test (α: 0.05; left tail) (***p-value < 0.01).

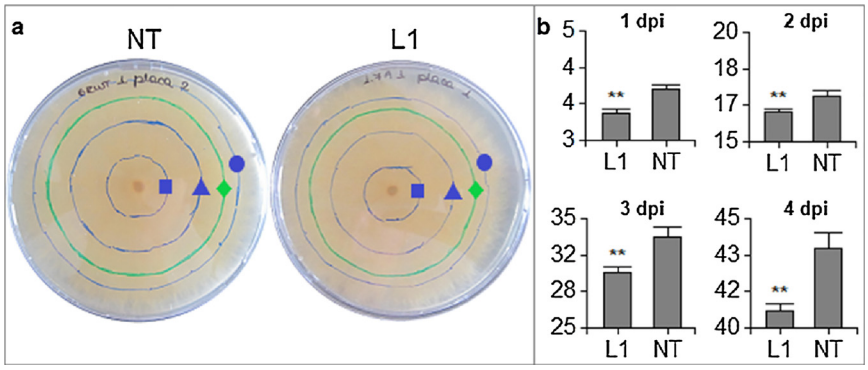

Fig. 4. *R. solani* mycelial growth inhibition by extracts of lettuce plants overexpressing Snakin-1 (a) Inoculated plate containing crude leaf extract from a NT (left) or L1 transgenic (right) plants. Circles on the plates represent the radial growth from the point of inoculation (center) across successive days in which the growth of mycelium was measured: 1 (■), 2 (▲), 3 (◆) and 4 (●) dpi. (b) Average area of *R. solani* mycelial growth on plates prepared with transgenic and NT extracts. The Y axis indicates the area (in cm^2) of mycelium radial growth at each dpi. Tukey's test (α: 0.05; $^{**}p$-value < 0.05) for mean comparison on each dpi. Bars indicate standard errors.

pathogens.

For some AMPs it was proposed that its mode of action is based on its interaction with the microbial membrane through hydrophobic or electrostatic forces, producing the disruption of the microbe membrane (Nguyen et al., 2011). Snakin-1 ability to disrupt the integrity of cell membranes was evaluated by Kuddus et al. (2016), who proved that the recombinant peptide expressed in *Pichia pastoris* produces hemolysis in erythrocytes as well as permeabilization of the outer membrane and depolarization of the cytoplasmic membrane of *E. coli*.

The *in vitro* activity of Snakin-1 (purified from potato) against bacteria and fungi has been widely reported (Berrocal-Lobo et al., 2002; López-Solanilla et al., 2003; Segura et al., 1999). In this work, we demonstrated the antimicrobial activity of Snakin-1 against *R. solani* growth by the action of the L1 transgenic plant crude extracts that inhibited the mycelial radial growth. This result showed that the potato peptide Snakin-1 expressed in lettuce maintains its activity *ex vivo*.

To corroborate the antimicrobial activity of Snakin-1 *in vivo,* an assay was carried out by growing concentric lines of seedlings in Petri dishes that were inoculated with *R. solani* in the center. All the transgenic lines expressing *snakin-1* gene were more protected against *R. solani* than the non-transgenic control line, since they had a greater number of asymptomatic seedlings. Unfortunately, this *in vivo* assay was not suitable to evaluate the response of the lines against *S. sclerotiorum* because the aggressiveness of this pathogen was too severe for the seedlings (data not shown). *S. sclerotiorum* aggressiveness on seedlings was also observed in challenge assays in *Arabidopsis thaliana* expressing HaGLP1, a germin-like defense protein from sunflower (Beracochea et al., 2015).

In order to evaluate if the plant protection conferred by Snakin-1 expression against *R. solani* was maintained in adult plants and whether it was extensive against *S. sclerotiorum* we performed challenge assays in greenhouse. Then, transgenic and NT plants were grown in pots in a greenhouse and inoculated at the rosette growth stage. All transgenic lines showed a statistically significant defensive response against *R. solani*. In addition, transgenic lines showed *in vivo* protection against *S. sclerotiorum* on adult plants, being statistically significant for two of the three lines.

Since it was suggested that Snakin/GASA proteins participate in stress responses by regulating the redox homeostasis (Nahirñak et al., 2012b) we analyzed the effect of Snakin-1 overexpression on the levels of ROS in lettuce leaves. Transgenic lines L2 and L3 showed a lower accumulation of O_2^- and H_2O_2 in comparison with NT plants. These findings are in agreement with observations in silenced *snakin-1* potato plants where the redox balance was altered showing higher levels of ROS (Nahirñak et al., 2012a). Accordingly, transgenic *Arabidopsis thaliana* plants overexpressing GASA4 suppressed the accumulation of H_2O_2 and nitric oxide in wounded leaves (Rubinovich and Weiss, 2010). In this context, we speculate that Snakin-1 could be participating in biotic stress responses to some extent through redox homeostasis, promoting a favorable redox environment that enables the host plant to cope with pathogen attack during the early stages of infection. Biotrophs complete their life cycle by feeding on living cells and it has been suggested that the effective defense against them is due in part to programmed cell death and to the activation of the defense response by the SA-dependent pathway (Glazebrook, 2005). In contrast, necrotrophs kill the host cells and feed on their contents so the production of ROS at the site of infection during the hypersensitive response (HR) would facilitate infection and host colonization of them. In recent years it has become apparent that necrotrophs could actively contribute to increasing the levels of ROS in the plant and even take advantage of the host response (Heller and Tudzynski, 2011).

This possible explanation of Snakin-1 antifungal activity mediated by a lower ROS accumulation would not explain line L1 results. The line L1 showed a statistically significant protection against *R. solani* and *S. sclerotiorum* in challenge experiments performed in greenhouse (Figs. 6 and 7) but ROS levels similar to the NT line (Fig. 3). This suggests that Snakin-1 may be acting through several molecular mechanisms in a coordinated manner, one of which could be the regulation of ROS state but not the only one.

Although the three transgenic lines consistently showed a measurable increased tolerance to both pathogens, they display some diverse degree of response in the different assayed experiments and conditions.

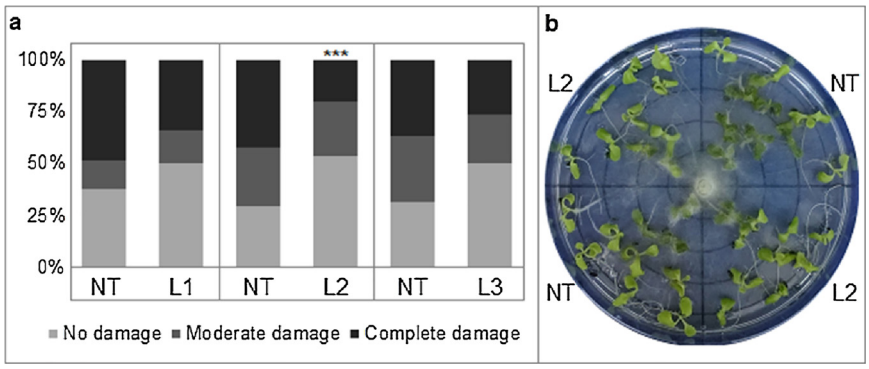

Fig. 5. Results of infection assays on seedlings challenged against *R. solani*. (a) Ratios of undamaged, moderately damaged and completely damaged seedlings in each line. Pearson's χ^2 test between seedlings of each transgenic line and the corresponding group of NT seedlings, considering the number of damaged, moderately damaged and completely damaged plants in contingency tables ($^{***}p$-value < 0.01). (b) Effects produced by *R. solani* in NT and transgenic L2 seedlings.

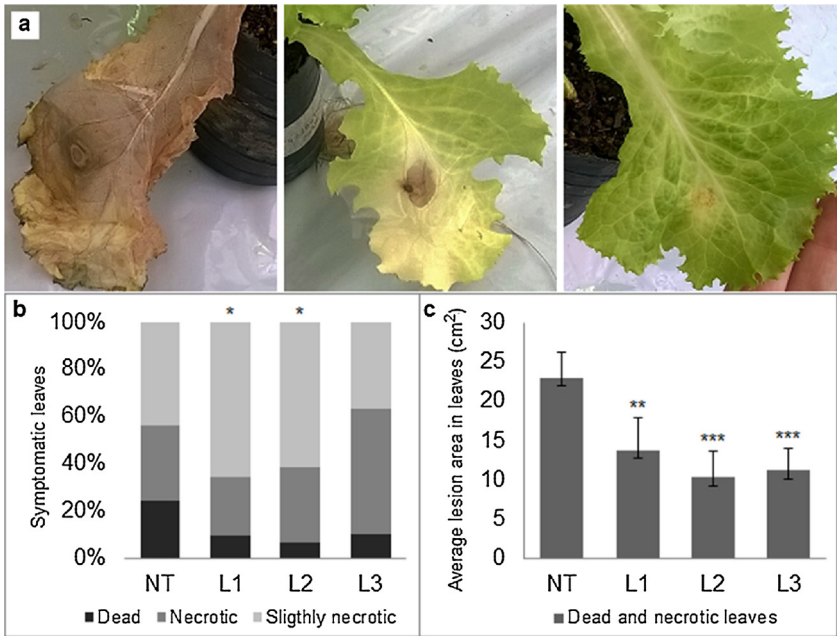

Fig. 6. Lesions produced by *R. solani* in lettuce leaves after 14 dpi. (a) Symptomatic leaves inoculated with an *R. solani* agar plug: slightly necrotic (left), necrotic (center) and dead (right). (b) Ratios of slightly necrotic, necrotic and dead leaves in each line. Pearson's χ^2 test between each transgenic line and the NT line, considering the number of slightly necrotic, necrotic and dead leaves (*p-value < 0.1). (c) Average lesion area (cm^2) in necrotic and dead leaves. Student's t-test (α: 0.05) indicating significant differences (***p-value < 0.01; **p-value < 0.05) from each line. Bars indicate standard errors.

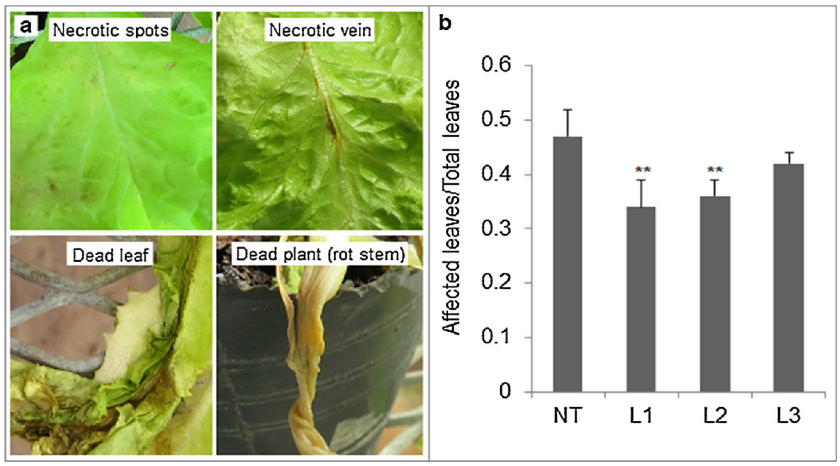

Fig. 7. Leaves affected by *S. sclerotiorum*. (a) Symptoms observed in leaves at 8 dpi. (b) Average ratio number of affected leaves/number of total leaves. Student's t-test (α: 0.05; left tail) for independent samples indicating significant differences (**p-value < 0.05) between each transgenic line and the NT line. Bars indicate standard errors.

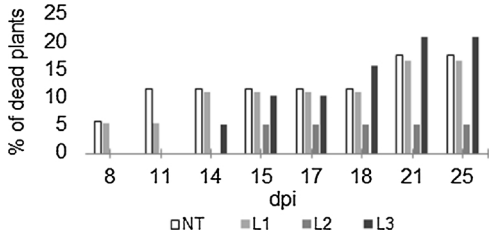

Fig. 8. Plants killed by *S. sclerotiorum*. Percentage of dead plants, from 8 to 25 dpi, according to the number of inoculated plants.

These differences may be due to the number of gene copies, their insertion site and their flanking regions that could be affecting the transgene temporal or tissue expression pattern. More studies are needed to clarify these points.

In conclusion, our findings support that the foreign expression of Snakin-1 plays a role in broad-spectrum defense responses in lettuce although the complete understanding of the molecular mechanisms by which this peptide may contribute to pathogen resistance remains unknown.

The overexpression of Snakin-1 in crop species might be useful not only for pathosystems for which partial resistance is the only kind of resistance known nowadays, but also for cases in which major resistance genes are available, but a broader-spectrum and a more durable resistance is still needed. Moreover, since no conspicuous morphological or developmental alterations seem to derive from Snakin-1 expression, this gene might also be conceived as a valuable biotechnological tool to be used alone or in combination with other antifungal proteins from different species, for the engineering of disease resistance in lettuce.

Funding

This work was supported by INTA (Instituto Nacional de Tecnología Agropecuaria), Argentina (grant numbers PNHFA1106073, PNBIO1131023 and PNBIO1131024). F. Darqui received a CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina fellowship.

Declarations of interest

None.

Acknowledgments

We thank V. Beracochea for her suggestions on ROS detection protocols and Dr. M. Rivero for her valuable assistance with the English text. We also thank Drs. N. Almasia and V. Nahirñak for critically reading this manuscript and helpful discussions.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.07.017.

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