Increased Tolerance to Wheat Powdery Mildew by Heterologous Constitutive Expression of the *Solanum chacoense snakin-1* Gene

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Abstract: Great efforts are currently being devoted to studying the use of transgenes to confer resistance to phytopathogenic fungi. Snakin-1 is a broad-spectrum antimicrobial peptide isolated from *Solanum* that is active *in vitro* against bacteria and fungi. Recently, it was reported that overexpression of the *snakin-1* (*SN1*) gene in transgenic potato plants enhanced resistance to *Rhizoctonia solani* and *Erwinia carotovora*. In this work wheat transgenic plants that constitutively expressed the *S. chacoense SN1* gene were challenged with *Blumeria graminis* f.sp. *tritici*. Enhanced resistance to the pathogen was observed in two transgenic lines in which the development of the disease was delayed and reduced compared with the wild type variety ProINTA Federal. An association between high resistance to the pathogen and a high level of snakin-1 transcripts in the plant was observed. This is the first report on *SN1* gene expression in Gramineae and its effects on wheat powdery mildew development.

Keywords: antimicrobial peptides; *Blumeria graminis* f.sp. *tritici*; genetic transformation; *snakin-1*; wheat

Wheat is a primary crop for basic human food and in 2009 worldwide production reached about 682 mil t (FAOSTAT). Wheat diseases are key production constraints that affect yield and quality. Powdery mildew caused by *Blumeria graminis* f.sp. *tritici* is considered one of the most damaging for this crop in the world (Liu *et al*. 2001; Lillemo & Skinnes 2006; Mikulová *et al*. 2008; Zhang *et al*. 2008; Chen *et al*. 2009), and has caused yield losses ranging from 13 to 34% (Liu *et al*. 2001; Lillemo & Skinnes 2006; Zhang *et al*. 2008) despite control with fungicides and crop rotation. Race-specific resistance genes have been widely used in wheat breeding programs to control this disease, but the selection pressure exerted by new cultivars results in the rapid emergence of new pathotypes with the corresponding virulence genes (Liu *et al*. 2001). Adult plant resistance retards infection, growth and reproduction of *B. graminis* f.sp. *tritici* in adult plants but not in seedlings (Chen *et al*. 2009), and is more durable than race-specific resistance. It is also called slow mildewing (Shaner 1973) and partial resistance (Hautea *et al*. 1987). Most genetic engineering work on wheat resistance to fungal pathogens has focused on the overexpression of single sequences that encode antimicrobial proteins (Patnaik & Khurana 2001; Sahrawat *et al*. 2003). Most of these genes belong to the so called pathogenesis related (PR) group, such as glucanases and chitinases. Other antimicrobial peptides (AMPs), like

defensins and thionins, have shown important antibacterial and antifungal activities *in vitro* as well as *in vivo* (Silverstein *et al*. 2007; Tavares *et al*. 2008). Transgenic plants carrying different defence genes expressed enhanced resistance to a variety of fungal and bacterial pathogens (Grover & Gowthaman 2003). Snakin-1 (SN1) is an AMP originally isolated from *Solanum tuberosum* cv. Desirée that has a broad antimicrobial action spectrum and is active *in vitro* against important pathogens such as *Clavibacter michiganensis* subsp. *sepedonicus*, *Fusarium solani*, *F. culmorum*, *Bipolaris maydis* and *Botrytis cinerea* (Segura *et al*. 1999). This cysteine-rich peptide has a conserved 60 amino acid carboxyl-terminal domain containing 12 cysteine residues. It shows similarity to members of the GAST (Gibberellic Acid Stimulated Transcript) family from tomato and GASA (Gibberellic Acid Stimulated in *Arabidopsis*) family from *Arabidopsis*. For this reason, snakin-1 has been classified as a new snakin/GASA family together with snakin-2 (Berrocal-Lobo *et al*. 2002). Recently, enhanced resistance to *Rhizoctonia solani* and *Erwinia carotovora* in transgenic potato plants overexpressing the *S. chacoense SN1* gene has been reported (Almasia *et al*. 2008). In this study, we demonstrate that constitutive expression of the *S. chacoense SN1* gene in wheat plants leads to enhanced resistance against *B. graminis* f.sp. *tritici*.

MATERIAL AND METHODS

Plasmid vectors and genetic transformation of wheat

Two vectors, each of them containing the *SN1* or *bar* gene sequences were used in co-bombardment transformation experiments. Plasmid pDM302 contains the promoter, first exon and first intron of the rice *Actin1* gene, the *bar* coding sequence and *nos* terminator sequence (Cao *et al*. 1992). The *bar* gene confers resistance to the herbicide phosphinothricin. The second vector, pUBI-cSN1, was constructed by replacing the *gusA* coding gene sequence present in the vector pDMC202 (McElroy *et al*. 1995) by the 295 base pairs (bp) coding sequence of the *S. chacoense SN1* gene without the intron. The maize ubiquitin promoter was sub-cloned upstream to the *SN1* coding sequence. The nopaline synthase gene terminator sequence of *Agrobacterium tumefaciens*, present in the original pDM202, was left without modification in pUBI-cSN1. Scutella of genotypes Bobwhite 1, ProINTA Federal, SH9856 and SH9826, grown in a growth chamber under 18/15°C day/night thermoperiod and 16/8 h photoperiod at a light intensity of 190 µmol/m^2s, were dissected from immature embryos and used for gene transfer following the biolistic procedure described in Pellegrineschi *et al*. (1999).

Molecular characterisation of transgenic plants

Plant genomic DNA was extracted from leaf tissue according to Dellaporta *et al*. (1983). The presence of the *bar* and *SN1* genes in the *in vitro* regenerated plants were analyzed by PCR. The specific primers used for gene amplification were: bar-forward (5'TGCACCATCGTCAACCACTA3'), bar-reverse (5'ACAGCGACCACGCTCTTGAA3'), SN1-forward (5'AGAAAAAATGAAGTTATTTCTATTAACT3') and SN1-reverse (5'ATCCTCAAGGGCATTTAGACTT3'). The PCR reaction was carried out in a final volume of 25 µl with 20–50 ng wheat genomic DNA. The plants carrying the transgene *SN1* were tested by RT-PCR using the Access RT-PCR System kit (Promega, USA). For Southern blot analyses, 20 µg DNA extracted from leaf samples of each plant (Saghai-Maroof *et al*. 1984) were digested overnight with *Bam*HI and the digestion products were separated by agarose (1% w/v) gel electrophoresis. DNA was transferred to a positively charged nylon membrane, and hybridized to digoxigenin (DIG)-dUTP labelled probes (F. Hoffmann-La Roche Ltd., Switzerland). For quantitative RT-PCR, total RNA was extracted from leaf tissues with Trizol (Invitrogen, USA). The RNAs were treated with RQ1 RNase-free DNase (Promega, USA). First strand complementary DNA (cDNA) was synthesized with oligo $(dT)_{18}$ as a primer and SuperScript III Reverse Transcriptase (Invitrogen, USA). An Icycler IQ Real – Time Detection System (BioRad, USA) was used. The wheat putative chromosomal condensation factor *TaCCF*, was used as a reference gene (Stephenson *et al*. 2007). The *SN1* transcript was amplified with the primers qSN-forward (5'AACTCTGCTTTTGGTCACTCTTG3') and qSN-reverse (5'AGTTCCAGAAGGCACACATTTG3'). The detection of products was performed using the IQ SuperMix PCR kit (BioRad, USA). The PCR cycling condition comprised one cycle at 95°C for 5 min, followed by 45 cycles at

95°C for 20 s and 60°C for 40 s. A melting curve was generated by the equipment to assure the specificity of the amplification reaction. For each sample the reaction was carried out in three replicates. The statistical analyses of the results were performed with the Relative Expression Software Tool REST© (Pfaffl *et al*. 2002).

Infection with *B. graminis* f.sp. *tritici*

Transgenic plants of progenies expressing the *SN1* gene were challenged with a local *B. graminis* f.sp. *tritici* isolate asexually propagated on the Argentinean susceptible cultivar Sureño. Primary leaf segments of 3 cm were placed in Petri dishes on water-agar medium (0.6%, w/v) supplemented with 20 mg/l benzylaminopurine (pH 5.7) to delay tissue senescence. Each Petri dish contained leaf samples of transgenic plants expressing *SN1*, transgenic plants not expressing *SN1* and non transgenic (wild-type) plants used as controls. Inoculation was carried out with fresh conidia by shaking infected plants at the top of a cardboard pipe of 1 m height on the open Petri dishes placed at the pipe bottom. This procedure assures a homogenous distribution on leaf tissues of 80 to 120 conidia/cm^2. Inoculated samples were incubated in a chamber at 18°C under continuous light (90 μmol/m^{2}s). The number of developing fungus colonies was counted 6 days after inoculation under a stereoscopic microscope (20×). ANOVA was performed using the InfoStat software version 2010 (Di Rienzo *et al.* 2010) using a random complete block design. Each Petri dish was regarded as a block. The leaf area covered by the pathogen was estimated at 6, 7, 8 and 12 days after inoculation by image analysis using the JMicroVision software program (Roduit N., JMicroVision v1.2.5). The extent of the infected area was considered a quantitative measure of disease severity.

RESULTS

Transformation efficiency and molecular characterisation of SN1 wheat transgenic plants

One hundred and thirteen plants were recovered from transformation experiments, and the presence of the *bar* gene was confirmed by PCR. Amongst these, a specific 295 bp DNA fragment was also amplified in 90 plants indicating the simultaneous presence of the *SN1* transgene. Transformation efficiency was 2% (No. of plants containing *bar* gene/No. of embryos bombarded × 100). The co-transformation efficiency was 79.6% (No. of plants containing the *bar* and *SN1* genes/No. of plants containing the *bar* gene × 100). Forty one primary transgenic plants (T_0) expressed the *SN1* transcript. Transgene expression in 8 of them was silenced in the T_1 or T_2 generations but it remained stable in the rest of the T_0 plants through the T_2 and T_3 generations. Southern blot analysis fully confirmed PCR results and showed that the number of inserted copies varied across the transgenic lines from 12 to approximately 20 per genome (see Figure 1).

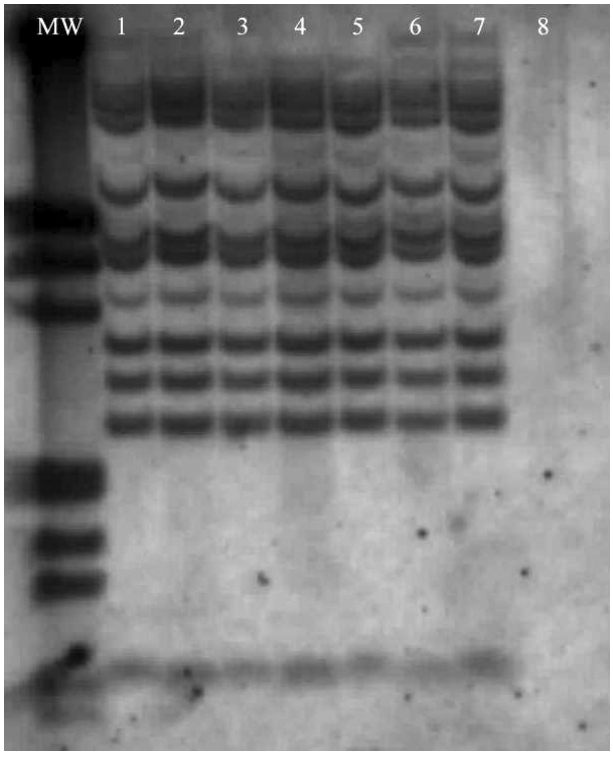

Figure 1. Southern blot analysis of T_2 plants derived from transgenic sn389 line; the genomic DNA was digested with *Bam*HI that cuts between the *SN1* coding sequence and the nopaline synthetase gene terminator; each band is considered an insertion site and approximately 15 insertion sites were present in sn389; all sn389 plants have the same gene insertion pattern, as expected; lanes 1 to 7, different plants of the sn389 line; lane MW: DNA Molecular Weight Marker III, Digoxigenin labeled; lane 8: wild type ProINTA Federal control

Enhanced resistance of SN1 wheat transgenic plants to powdery mildew

The inoculation experiments were carried out on plants derived from selfing 33 independent T_0 events. Twenty six progeny families showed segregation of the multiple transgene copies, while 7 did not. T_2 progeny derived from these 7 T_0 plants expressing *SN1* gene transcripts, were challenged with *B. graminis* f.sp. *tritici*. Among these T_2 progeny families, one transgenic line, named sn389, behaved as resistant when compared to the wild type control, and to other transgenic lines that did not express the transgene. The progenies of the remaining 19 T_0 plants that were still segregating at the T_2 generation, were evaluated at the T_3 and T_4 generations, when homozygous lines were achieved. A second resistant transgenic line, named sn178, was identified in T_4. Both resistant lines, sn178 and sn389, were obtained from the variety ProINTA Federal. The number of developing mildew colonies was recorded 6 days after inoculation. Statistically significant differences in independent experiments were found after comparing ProINTA Federal non transgenic controls against each of the resistant transgenic lines: $F_{1,22} = 8.43$; $P < 0.01$ for sn178 and $F_{1,6} = 50.34$; $P < 0.001$ for sn389. The growth of colonies also showed differences between transgenic and non transgenic plants. These differences increased over time (Figure 2). Thus, fungal development was clearly affected on the resistant lines both in terms of number and size of colonies (Figure 3).

SN1 expression correlates with fungal resistance

Quantitative RT-PCR transcript analyses of the resistant transgenic lines were performed. A transgenic line named sn305, having a behaviour similar to ProINTA Federal against the fungus (Figure 2), was considered the control reference line for *SN1* transcription comparisons. Significant differences ($P < 0.001$) were established between the transcription levels of the sn305 line and the two transgenic resistant lines, sn178 and sn389, that, respectively, expressed 4.226 and 8.532 fold more transcripts than sn305. Thus, an association was found between resistance to the pathogen measured on leaf segments and higher levels of snakin-1 transcripts in plant leaves.

DISCUSSION

The transformation efficiency obtained in our study (2%) was similar to that observed in wheat by Vasil *et al.* (1993), Oldach *et al.* (2001) and MacKintosh *et al.* (2006). However this efficiency might be considered a limitation if the study of many transgenes is tackled with limited operational capacity (Dahleen *et al.* 2001). To illustrate this issue, it is interesting to mention that only 41 out of the 90 transgenic plants (T_0) achieved in this work, expressed the *SN1* transcript. Of these, 3 were sterile and 8 were silenced in later generations. Consequently the final transformation efficiency of transcriptionally active transgenic

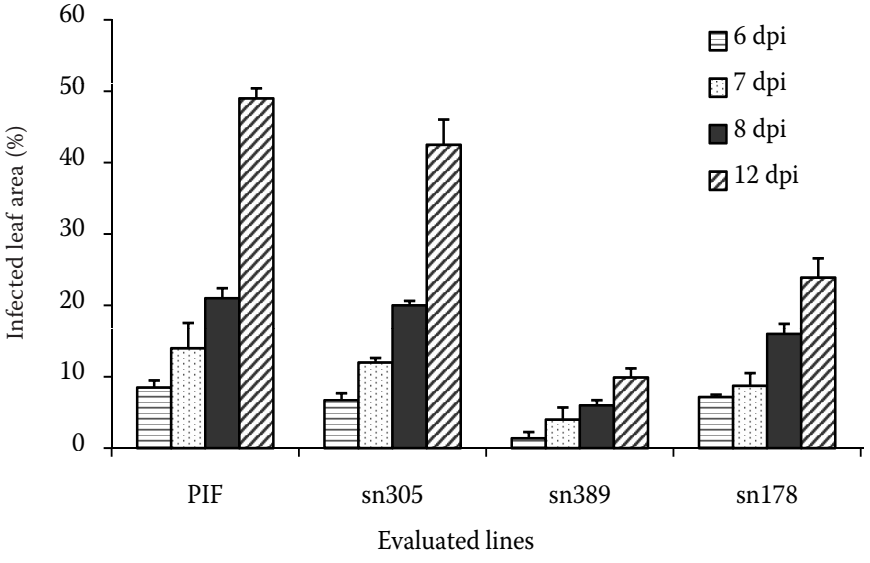

Figure 2. Evolution of *Blumeria graminis* f.sp. *tritici* growth on infected leaf segments; the percentage of infected area was calculated by image analysis using JMicroVision software; the sn389 and sn178 are selected resistant lines; ProINTA Federal and sn305 are susceptible lines; the transgenic sn178, sn389 and sn305 lines expressed *SN1* gene at different transcription levels; PIF: ProINTA Federal; dpi: days post infection; the error bars indicate standard deviation measured in two replicates

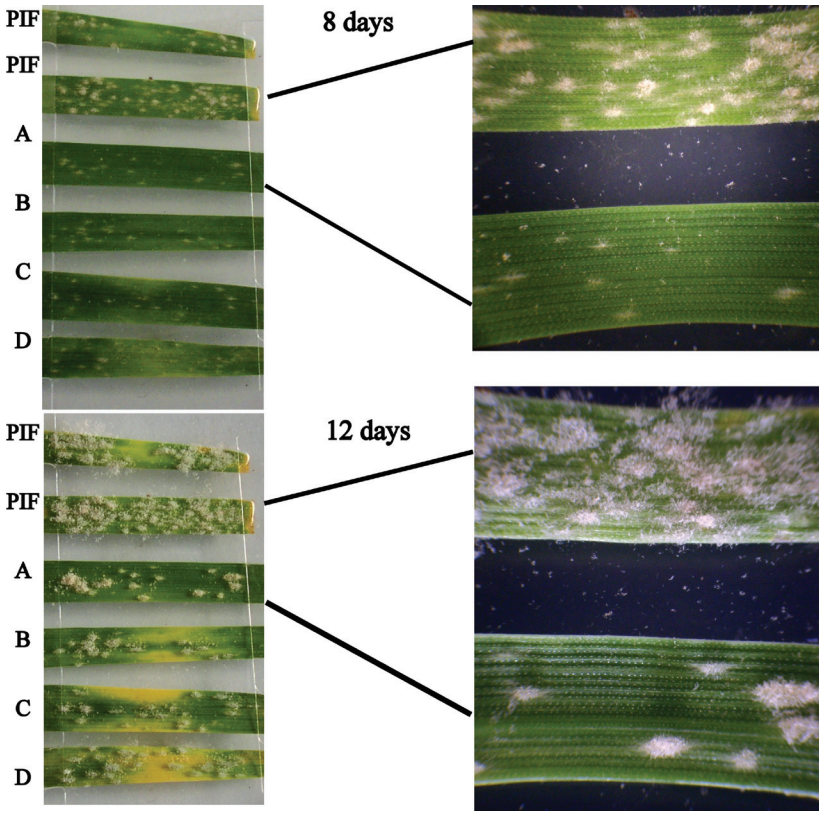

Figure 3. Development of *Blumeria graminis* f.sp. *tritici* 8 and 12 days post inoculation on the same leaf segments; PIF: wild type genotype ProINTA Federal; A, B, C and D: transgenic plants of genotype sn389 expressing snakin-1

plants was reduced to nearly one third (33/90) of the *SN1* T_0 plants achieved.

The presence and transcriptional expression of the transgenes were confirmed by PCR and RT-PCR through the T_0–T_4 generations. Transgene expression in eight of the T_0 events was silenced in the T_1 or T_2 generations. This result agrees with those of Alvarez *et al.* (2000), Anand *et al.* (2003), Makandar *et al.* (2006) and Chen *et al.* (2008), who also observed silencing during the early generations of selfing in transgenic plants. Gene silencing is usually associated with integration of transgenes in multiple copies and to sequence homology between a transgene and an endogenous gene. According to our Southern blot results, most of the *SN1* transgenic plants had a high number of insertion sites, and silencing events could be expected by this fact.

Plants were evaluated for reaction against *B. graminis* f.sp. *tritici*. Disease severity was determined by scoring the number of developed colonies by leaf area, at periods of 6, 7, 8 and 12 days post infection, taking photographs of the leaf segment and quantifying diseased areas with JMicroVision software. The development of the disease in terms of both colony numbers and infected area, was delayed in the transgenic lines sn389 and sn178 as compared to the wild type variety control ProINTA Federal. During the 6–12 days post infection period analysed, the progress of disease development was significantly slower in the transformed lines. It is worth mentioning that, to our knowledge, this is the first report regarding potato *SN1* constitutive expression in wheat or other gramineae species.

In wheat, for mildew and other diseases, many genes have been identified that confer different resistance levels, and that are available for breeding programs. Nevertheless, the high resistance level given by these genes is usually not durable, due to pathogen population evolution (Lillemo & Skinnes 2006). Protection observed in the sn398 and sn178 lines was similar to that conferred by conventional partial resistance genes in terms of reduction of pustule size, reduced production of spores and longer latency period of disease development (Hÿckelhoven 2005; Mikulová *et al.* 2008). This resistance behaviour leads to a significantly reduced damage level when compared to a susceptible host plant by retarding the pathogen reproductive cycle. Likewise, the smaller amount of available fungus spores in the field along the cropping period leads to a delay in the spreading of the disease on susceptible cultivars. From an

epidemiological point of view, the resistance observed in sn389 and sn178, like that observed with conventional partial resistance, reduces the number of pathogen generations during the cropping season as well as inoculum production. As reported here, no total or complete resistance against fungal pathogens was reported to occur after transformation with any other single gene encoding antifungal proteins in wheat. Although there is evidence in other systems that the constitutive expression of this protein can confer protection to a wide spectrum of pathogens, including bacteria, the results reported here cannot yet be extended to other pathogenic systems of wheat. The snakin-1 transgenic resistant lines obtained in this study will be challenged with other pathogens to study this point.

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