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ORIGINAL ARTICLE

Expression of a sphingomyelinase-coding gene from *Trichoderma harzianum* conferred bacterial tolerance in tobacco

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Abstract

Diseases caused by bacteria are an important and widespread constraint, occurring in almost all crops, including vegetables, pulses, cereals, ornamentals, fruit and forages. Although several strategies have been developed to obtain disease resistance in plants using genetic engineering, few studies have effectively demonstrated the control of bacterial diseases. It has previously been reported that a gene encoding a sphingomyelinase (*ThSMase*), identified in *Trichoderma harzianum*, is up-regulated during biocontrol against phytopathogens, and this may have biotechnological applications. SMases are involved in multiple cellular functions, including the immune response against pathogens. We hypothesized that the expression of this gene from fungi in transgenic tobacco could generate resistance to plant pathogens. *ThSMase* was cloned under control of the double CaMV 35S promoter plus a leader sequence from alfalfa mosaic virus (AMV), and stably introduced and expressed in tobacco. Reverse transcription-quantitative PCR analysis revealed that the *ThSMase* gene was expressed at similar levels in all transgenic lines tested. Our results showed no statistically significant difference in susceptibility after challenging transgenic and non-transgenic lines with the fungus *Sclerotinia sclerotiorum*. However, transgenic tobacco plants revealed a significant resistance to the bacterium *Pseudomonas syringae* pv. *tabaci*, and a tolerance to *Xylella fastidiosa*. Our results demonstrated the strong potential of *ThSMase* in biotechnological processes such as molecular breeding of the plants.

KEYWORDS
biological control, genetic engineering, *Pseudomonas syringae*, *Sclerotinia sclerotiorum*, transgenic plants, *Xylella fastidiosa*

1 | INTRODUCTION

Agriculture is one of the most important activities for the economic, social and technological development of society. However, plant diseases affect efforts to increase crop production and productivity, endangering economies and food security (Nelson, 2019). Diseases caused by bacteria are an important and widespread constraint, occurring in almost all crops, including vegetables, pulses, cereals, ornamentals, fruit and forages (Sundin et al., 2016). Bacterial diseases are difficult to identify and to control, and few pesticides are available for their effective management (Borkar & Yumlembam, 2017).

Plant disease management employs several strategies involving chemical and biological control of pathogens; it may also involve the use of resistant or tolerant genotypes manipulated by both conventional and molecular breeding. Although several strategies

using genetic engineering have been developed to obtain plant disease resistance (Collinge & Sarrocco, 2021), only a few studies have demonstrated the effective control of bacterial diseases (Sundin et al., 2016). Most disease-resistant transgenic crops commercially available so far are resistant to viruses (ISAAA, 2021).

Trichoderma species have the capacity to antagonize pathogenic fungi by the production of antibiotics and/or hydrolytic enzymes, competition for nutrients and mycoparasitism (Druzhinina et al., 2011; Vinale et al., 2008). In addition, they have beneficial effects on plants due to the fact that they can colonize roots and trigger systemic resistance against bacterial and fungal pathogens (Alfiky & Weisskopf, 2021; Hermosa et al., 2012). These abilities may represent effective strategies to be used in agriculture for reducing plant diseases, increasing crop yield and reducing economic losses.

Due to their potential as a biotechnological tool, *Trichoderma* genes have been expressed in plants, aiming to introduce tolerance to both biotic and abiotic stresses (Dana et al., 2006; Kaur et al., 2021; Kumar et al., 2009; Montero-Barrientos et al., 2010; Shah et al., 2009; Silva et al., 2019). Until recently, the insertion of genes encoding *Trichoderma* cell wall-degrading enzymes was the main strategy used to evaluate the possibility of improving plant resistance (Nicolás et al., 2014; Silva et al., 2019). However, we studied genes expressed in *Trichoderma harzianum*, during biological control of fungi, and identified various genes with potential biotechnological value; these genes encoded proteins that function as transporters, or in hydrolytic activity, adherence, appressorium development and pathogenesis (Vieira et al., 2013). Among the up-regulated genes, the gene encoding an acid sphingomyelinase was identified.

Sphingomyelinase (SMase), also known as sphingomyelin phosphodiesterase, belongs to the metallophosphatase superfamily; it cleaves sphingolipids, a class of structural membrane lipids involved in multiple cellular functions, such as regulating protein intake, cell proliferation and differentiation (Hannun & Obeid, 2008; Marchesini & Hannun, 2004). In addition, it can act as a biochemical marker, initiating programmed cell death in plants (Chen et al., 2006). SMases are found in animals and microorganisms, and are catalogued according to their activity and optimal pH: acidic SMase, secretory SMase, neutral, Mg^{+2}-dependent SMase and alkaline SMase (Goni & Alonso, 2002; Goni et al., 2012). In mammals, SMases are found in cells and body fluids, functioning as a secondary messenger inducing an apoptotic response (Gorelik et al., 2016; Perrotta et al., 2015), and in the immune response against pathogens (Yu et al., 2009). It was discovered that some SMase-like enzymes are inactive against sphingomyelin, and instead can hydrolyse nucleoside diphosphates (NDP) and triphosphates (NTP), which may play a role in purinergic signalling (Airola et al., 2017). Thus, sequence homology with mammalian SMase might not be sufficient to predict substrate specificity, optimum pH or metal dependence (Airola et al., 2017). In bacteria, sphingomyelinases are secreted and function as toxins, acting in the breakdown of the host cell membrane (Matsuo et al., 1996).

On the basis of this information, it was decided to investigate the effect on plant pathogens of expressing the SMase-coding gene from *T. harzianum* (*ThSMase*) in tobacco plants (*Nicotiana tabacum*). We hypothesized that the expression of this gene could generate resistance to plant pathogens.

2 | MATERIALS AND METHODS

2.1 | Plasmid construct and plant transformation

The *ThSMase* gene from *T. harzianum* was synthesized by Epoch Life Science Inc. (GS52674-6; Sugar Land, TX, USA), using the *Arabidopsis thaliana* codon usage (GenBank accession for the *ThSMase*: EHK26001). The *ThSMase* coding region was cloned under the control of the 35S RNA promoter from cauliflower mosaic virus (35S CaMV) plus the alfalfa mosaic virus (AMV) enhancer, inserted between the *Nco*I/*Sac*I sites from the pCGCHI vector (Nunes et al., 2009). The *ThSMase* expression cassette was transferred to pCambia3300 using *Eco*RI and *Hin*dIII, generating the vector pSphingo (Figure 1a), which was used to transfect *Agrobacterium tumefaciens* EHA105. Tobacco plants (cv. Xanthi) were then transformed with the *Agrobacterium* using the leaf disk method, according to Horsch et al. (1985). The pSphingo vector contained the *bar* gene from *Streptomyces hygroscopicus* that encodes for the phosphinothricin N-acetyltransferase (PAT) enzyme, which confers tolerance to glufosinate ammonium (GA), and the presence of this protein was used to select genetically modified plants.

2.2 | Screening of transgenic events and progeny analyses

Plants were initially screened for expression of the *bar* gene. The presence of PAT was analysed using the GMO Trait Check LL Kit (Strategic Diagnostic Inc.). Leaf tissues (100 mg) were macerated in 300 μl of phosphate-buffered saline (PBS) for 15 s. Then, an immunostrip was inserted into the microtube and read after 10 min of incubation when positive results may be visible. The presence of the *ThSMase* transgene was verified by PCR (according to Lacorte et al., 2010), using the primer pair Sphingo973F (5′-TGGACAAGATGGGTTGGAGC-3′), and Sphingo1344R (5′-GCCATAACAGCATGAGAAGCA-3′) to amplify a 371 bp region within the *ThSMase* coding sequence. PCR conditions were 95°C (5 min) denaturation; 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min; and a final elongation cycle of 7 min at 72°C.

Seeds of the first generation (T_1) of self-pollinated plants were germinated and maintained at 25 ± 2°C, with 70%–90% relative humidity, under greenhouse conditions without additional illumination. Plants were analysed for the presence of the *ThSMase* transgene by PCR, as described. Pearson's chi-squared (χ^2) with Yates's correction was used to determine whether the observed segregation ratio was consistent with a Mendelian ratio of 3:1, at 95% level of confidence.

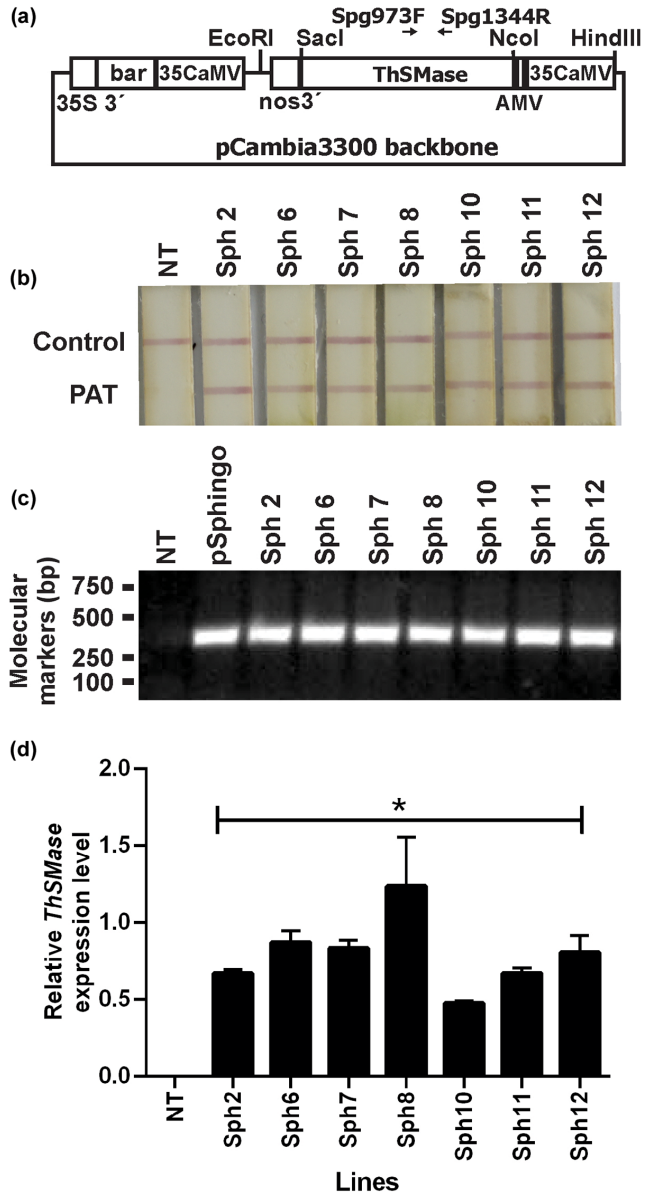

FIGURE 1 (a) Diagram of the vector pSphingo used to overexpress the *ThSMase* gene from *Trichoderma harzianum* in transgenic tobacco plants. The *ThSMase* gene was cloned under the control of the 35S RNA promoter from cauliflower mosaic virus (35S CaMV) plus the alfalfa mosaic virus (AMV) enhancer. The *ThSMase* expression cassette was cloned into the pCambia3300 vector, which contains the *bar* gene that confers tolerance to glufosinate ammonium, used for selection of transformed plants. Arrows indicate the position of primers used for PCR analyses. (b) Immunochromatographic analyses of transgenic tobacco plants for the expression of the *bar* gene (presence of phosphinothricin N-acetyltransferase [PAT] protein). (c) PCR analysis for detection of the *ThSMase* gene in transformed lines. (d) Results of reverse transcription-quantitative PCR for measuring the relative expression of the *ThSMase* gene in leaves of transgenic and nontransgenic (control) tobacco lines. Data represent means of three biological and three technical replications, where the control is considered as zero. The asterisk indicates a statistically significant difference in *ThSMase* expression of the transgenic lines compared to the control ($p < 0.05$, $n = 9$). There was no significant difference among transgenic lines. Bars are shown ± *SE*. NT: nontransgenic line

2.3 | Reverse transcription-quantitative PCR analysis

The transcription level of the *ThSMase* transgene was determined by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from young leaves using TRIzol (Invitrogen), and cDNA synthesized using 2 μg total RNA, according to the protocol described by reverse transcriptase Superscript VILO (Promega). RNA samples (2 μg) were treated with 2 U DNase I for 10 min at 37°C to eliminate any genomic DNA. After DNase I digestion, the samples were heated to 90°C for 5 min. Quantitative real-time PCRs were performed on a StepOnePlus Real-Time PCR cycler (Applied Biosystems) using a Platinum SYBR Green qPCR Super Mix-UDG w/ROX Kit (Invitrogen). To analyse the transcription levels of *ThSMase* in different transgenic lines, the primers SphingoRTF (5′-CAGCATGAGAAGCATCTTGC-3′) and SphingoRTR (5′-TTGGACACATGCCTATTGGA-3′) were designed using the software Primer3 Plus. Reactions followed the parameters described by Morgante et al. (2011). The relative transcription levels in different RNA samples were normalized with internal standard genes *EF1α*, using the primer pair EF1aRTF (5′-TGAGATGCACCACGAAGCTC-3′)/EF1aRTR (5′-CCAACATTGTCACCAGGAAGTG-3′) and 60S ribosomal protein L23a, using the primer pair L25RTF (5′-CCCCTCACCACAGAGTCTGC-3′)/L25RTR (5′-AAGGGTGTTGTTGTCCTCAATCTT-3′) obtained from Schmidt and Delaney (2010). Quantitative assays were performed in triplicate with three biological samples. The online real-time PCR Miner tool (Zhao & Fernald, 2005) was used to estimate the average cycle threshold (C_q) values and the qGENE software (Joehanes & Nelson, 2008) to evaluate the relative level of *ThSMase* expression.

2.4 | Inoculation of transgenic plants with pathogens

Transgenic plants were tested for resistance to *Sclerotinia sclerotiorum*, as described by Cunha et al. (2010). Briefly, a mycelial agar plug 2 mm in diameter was cut from the growing margins of a 2-day-old *S. sclerotiorum* culture and applied to the adaxial surface of a detached fully expanded leaf. Two leaves were inoculated per plant for five plants of each line. Symptoms were observed after 24 and 48 h and lesion area recorded. Leaves were kept in a plant growth chamber at 25 ± 2°C, 90%–100% relative humidity in the dark.

Transgenic lines were challenged with *Pseudomonas syringae* pv. *tabaci* (IBSBF 766) following the protocol described by Li et al. (2000). Briefly, bacterial suspension grown in medium 523 (Kado & Heskett, 1970) for 48 h at 28°C was centrifuged, resuspended in distilled water and adjusted to standard 7 of the McFarland scale (c.2.1 × 10^9 bacteria/ml) (McFarland, 1907). The suspension was infiltrated into the abaxial surface of detached leaves using a syringe without a needle. Two leaves were infiltrated per plant

from five plants of each line. Leaves were kept in chamber at 25 ± 2°C, 90%–100% relative humidity in the dark. Leaves were photographed every 7 days and the images were used to measure the infected area using ImageJ software (imagej.nih.gov/ij/). *P. syringae* was reisolated from lesions according to Stefanova et al. (2009). Briefly, the edge of leaf lesions was cut and macerated in PBS. The extract was striated in a plate containing semiselective medium (MSP-M; Mohan & Schaad, 1987), allowing the distinction between colonies of *Pseudomonas* species in a population of saprophytes. Chlorophyll loss in the lesion area was measured using a chlorophyll content meter model CCM-200 Plus GPS (Opti-Sciences, Inc.), reading a circular area of 9.5 mm diameter. The chlorophyll concentration index was obtained and compared to the control (inoculated with water) to calculate the chlorophyll loss.

Transgenic and nontransgenic plants were inoculated with *Xylella fastidiosa* subsp. *pauca* (strains 9a5c and Itápolis) according to Lopes et al. (2000). Briefly, *X. fastidiosa* was grown in PWG medium (Hill & Purcell, 1995) and suspended in PBS at a concentration with OD_{600nm} of 0.4 for pinprick inoculation. qPCR was performed 30 days after inoculation, according to Francis et al. (2006), to detect *X. fastidiosa* with a high degree of sensitivity and specificity. The primers used amplified a 221 bp unique region common to *X. fastidiosa* subsp. *pauca* strains. A plasmid (pJET-221) containing the same 221 bp qPCR product was sequenced and used to generate a standard curve for determining concentrations of *X. fastidiosa* in samples. The C_q 35.0 cut-off was used to determine whether a plant was positive for infection with *X. fastidiosa*, and titre determination (Francis et al., 2006).

All experiments were repeated twice with five replications.

2.5 | Statistics

The experiments were carried out in a completely randomized design. The results were analysed by analysis of variance (ANOVA) at $p < 0.05$ followed by Dunnett's test to compare between treatments. Correlation analyses (relative expression of the *ThSMase* gene versus lesion area caused by *P. syringae* or *S. sclerotiorum*) were calculated using Pearson's correlation coefficient at 95% confidence interval. All statistical analyses were implemented in Graphpad Prism v. 6.0 software.

3 | RESULTS

The leaf disk method was used to transform the tobacco (*N. tabacum*) plants using *A. tumefaciens* expressing an acidic sphingomyelinase-coding gene from *T. harzianum* (*ThSMase*). Seven transgenic lines were selected for the assays by the presence of the PAT enzyme (Figure 1b), and also the presence of the *ThSMase* transgene in PCR analyses (named as Sph2, Sph6, Sph7, Sph8, Sph10, Sph11 and Sph12; Figure 1c). All the plants were acclimatized and allowed to set seeds after 3 months. It was observed that all transgenic lines presented normal phenotypes (plant height and architecture, number of leaves, number of branches and number of flowers and fruits) when compared with nontransgenic plants. RT-qPCR analysis was carried out as an attempt to determine the cause–effect relationship between expression of the *ThSMase* transgene and the observed phenotype (disease resistance). The results showed that *ThSMase* was transcribed in leaves of all transgenic lines at levels that were statistically significantly higher than the nontransgenic control, but similar among transgenic lines (Figure 1d). Segregation analyses of the T_1 generation revealed that both *ThSMase* and *bar* transgenes co-segregated in a Mendelian fashion of 3:1 in all lines (Table 1) that were analysed further.

TABLE 1 Segregation analysis of the *ThSMase* transgene in the T_1 generation of self-fertilized transgenic plants

	ThSMase transgene		Ratio of 3:1	
Line	Positive	Negative	χ^2	*p*
Sph2	13	2	1.48	0.29
Sph6	12	3	0.42	0.65
Sph7	9	6	1.48	0.18
Sph8	13	2	1.48	0.29
Sph10	13	2	1.48	0.29
Sph11	13	2	1.48	0.29
Sph12	10	5	0.42	0.46

Leaves from transgenic and nontransgenic plants were inoculated with *P. syringae* pv. *tabaci*. The disease progress was monitored for a period of 14 days after inoculation (dai), and lesion area and chlorophyll loss were recorded. At 7 dai, significant differences in susceptibility were observed in transgenic lines compared to nontransgenic lines (Figure 2a). Nontransgenic leaves became widely affected by loss of chlorophyll. As disease progressed, it was observed that by 14 days the lesion area ranged from 28.5 to 43.6 mm^2 in the transgenic lines, while in the nontransgenic plants it was 358.6 mm^2 (Figure 2b). A statistically significant correlation was observed between the relative expression of the *ThSMase* gene and the lesion area caused by *P. syringae* at 7 dai (Pearson correlation $r = -0.796$, $p = 0.018$) and 14 dai (Pearson correlation $r = -0.783$, $p = 0.021$). *P. syringae* was reisolated from leaves; bright colonies were observed, with edges of light-yellow colour, which became orange after 72 h, changing the MSP-M medium from green to yellow. This confirmed the presence of *P. syringae* in the lesion areas. In addition, chlorophyll losses in the lesion areas were measured and, in the transgenic lines, ranged from 13% to 32%, while in the nontransgenic line it was 64% (Figure 2c).

Transgenic lines were challenged with *X. fastidiosa*, and when the plants were analysed by qPCR, the nontransgenic genotypes revealed that 100% of the plants were infected with the strain 9a5c, and 96.7% with the strain Itápolis (Figure 3). In contrast, the percentage of infected transgenic lines ranged from 25% to 83% with strain 9a5c and from 31% to 57% with strain Itápolis (Figure 3). Lines

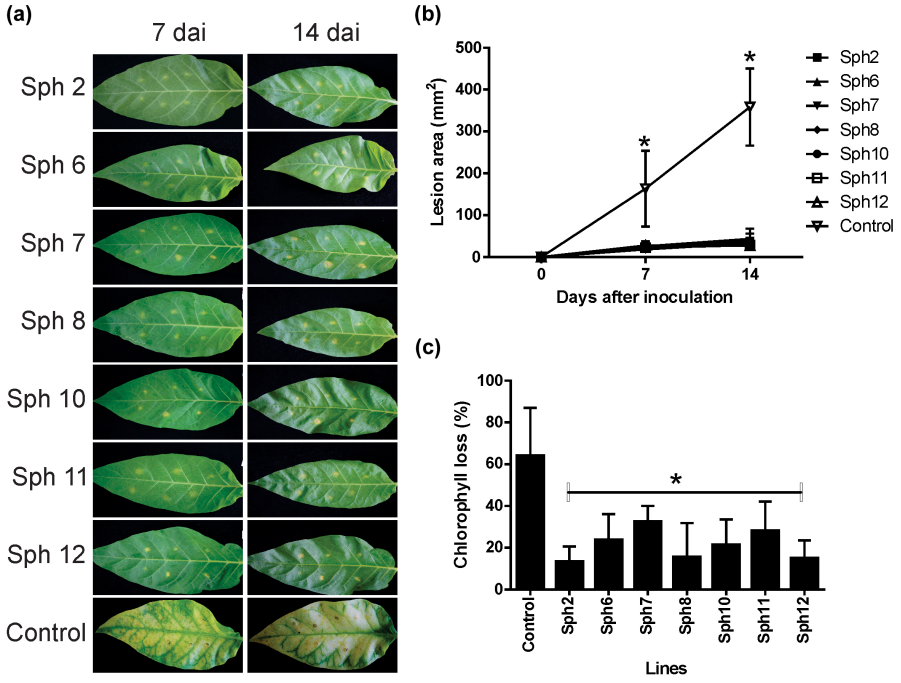

FIGURE 2 Resistant response of transgenic tobacco leaves expressing the *ThSMase* gene, inoculated with *Pseudomonas syringae* pv. *tabaci*. (a) Symptoms were observed 7 and 14 days after inoculation (dai). (b) Disease progress curve showing leaf lesion area with days after inoculation (dai). Asterisks indicate a significant difference between the lesion area of transgenic lines and the control. (c) Percentage loss of chlorophyll 14 dai in transgenic lines and the nontransgenic control. The asterisk indicates a significant difference in loss of chlorophyll between the transgenic lines and the control, but there was no difference among transgenic lines. $^{*}p < 0.05$, $n = 10$. Bars are shown ± *SE*

Sph2 and Sph12 did not show any statistically significant differences in infection by strain 9a5c compared to the control (Figure 3). No *X. fastidiosa* was detected in noninoculated plants.

Detached leaves of tobacco were inoculated with 2-mm-diameter agar plugs from the growing margins of 2-day-old *S. sclerotiorum* cultures, and lesion length was recorded 48 and 72 h after inoculation. Results showed that after 72 h the lesion area ranged from 423 to 844 mm^2 (average of 654 mm^2) in the transgenic lines, while in the nontransgenic plants it ranged from 405 to 1530 mm^2 (average of 922 mm^2; Figure 4). However, no statistically significant differences were observed among the transgenic and nontransgenic lines. No statistically significant correlation was observed between the relative expression of the *ThSMase* gene and the lesion area caused by *S. sclerotiorum* at 24 h (Pearson correlation $r = 0.333$, $p = 0.938$) or at 48 h (Pearson correlation $r = -0.211$, $p = 0.615$) after inoculation.

Segregating plants without the transgene, but that had undergone the transformation process, were also challenged with *P. syringae* pv. *tabaci*, *X. fastidiosa* and *S. sclerotiorum*, and gave similar results to the control (non-GM) plants (data not shown).

4 | DISCUSSION

Bacterial diseases in plants are difficult to control and are among the most critical biotic stress factors affecting plant development, crop yield and quality (Sundin et al., 2016). Management usually requires the combination of several complementary measures. Emphasis is on preventing the spread of the bacteria rather than on curing the plant (Ampatzidis et al., 2017). Integrated management measures for bacterial plant pathogens include cultural practices, chemical treatments, use of antagonistic or biological control products and implementation of strict quarantine (Sundin et al., 2016). However, the most effective low-cost strategy that could easily be adopted by farmers is the use of resistant genotypes (Lopes et al., 2012). Consequently, disease resistance has received high priority in many breeding programmes. Although traditional breeding plays an essential role, using the genetic resources of the centres of origin and diversity, molecular breeding could also be useful in generating broader and durable bacterial resistance.

Several biotechnological strategies have been used to produce transgenic plants resistant to both bacterial and fungal diseases (Dong & Ronald, 2019). Nevertheless, there are fewer cases of genetically engineered plants for bacterial resistance and most of the examples approved for commercial release are against viral (27 instances) and fungal (two instances) diseases (Dong & Ronald, 2019; ISAAA, 2021; Rooney et al., 2020).

SMases and SMase-like proteins have been found in arachnids, bacteria (*Corynebacteria* and *Arcanobacterium*) and fungi (*Aspergillus* and *Coccidioides*) (Cordes & Binford, 2006; Dias-Lopes et al., 2013). We have demonstrated the importance of the *ThSMase* gene in *T. harzianum*, which had previously been shown to be expressed during biocontrol of the plant pathogen *Fusarium*

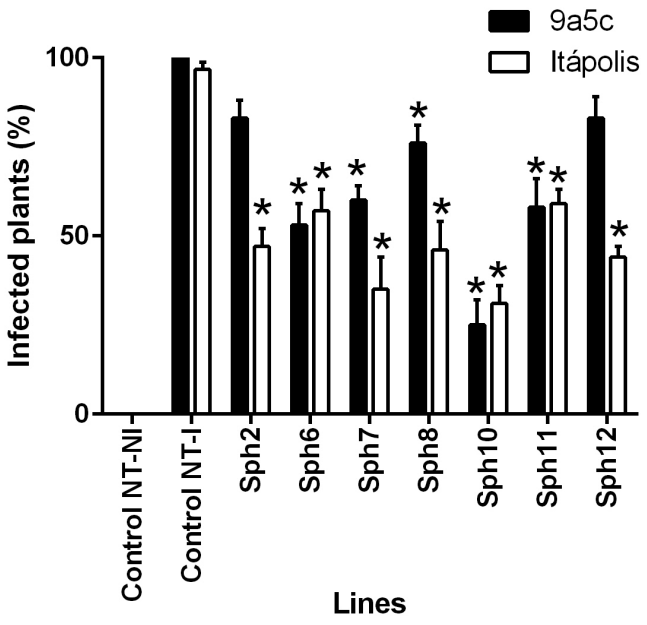

FIGURE 3 Response of the tobacco transgenic lines expressing the *ThSMase* gene, 30 days after inoculation with *Xylella fastidiosa* subsp. *pauca* (strains 9a5c and Itápolis). The number of infected plants was determined by quantitative PCR using a C_q 35.0 cut-off to indicate whether a plant was positive. NT = nontransgenic lines. NI =noninoculated. I = inoculated. The asterisks indicate a significant difference in the number of plants infected compared to the inoculated control (NT-I), $p < 0.05$, $n = 6$. Bars are shown ± *SE*

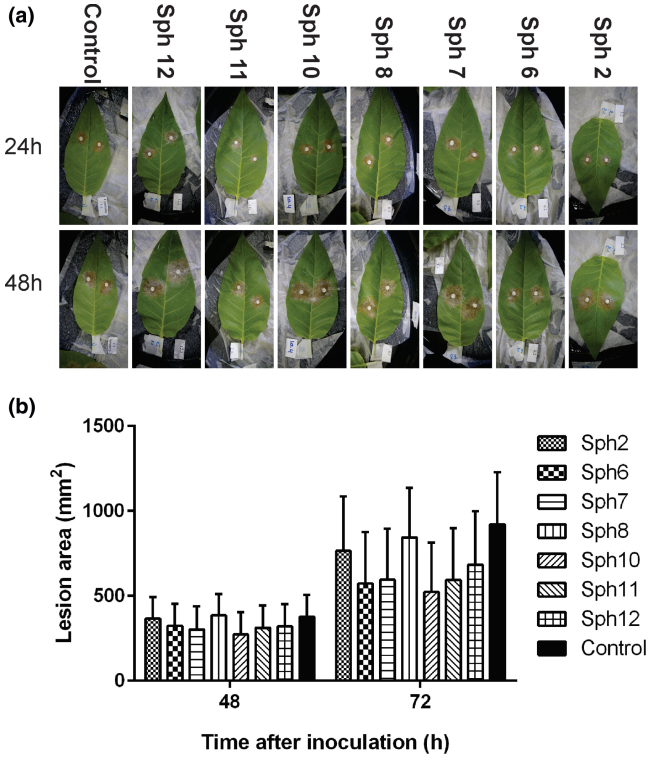

FIGURE 4 Response of transgenic tobacco leaves expressing the *ThSMase* gene and the nontransgenic control, inoculated with *Sclerotinia sclerotiorum*. Symptoms were observed 48 and 72 h after inoculation (a) and the progress of the disease was determined by lesion area (b). No statistical differences were observed between 48 and 72 h ($p < 0.05$, $n = 10$). Bars are shown ± *SE*

solani (Vieira et al., 2013). In the light of our results, SMases from *Trichoderma* may open new perspectives for improving plant resistance to biotic stresses without altering other characteristics of the plants.

When transgenic tobacco plants expressing *ThSMase* were challenged with the pathogenic fungus *S. sclerotiorum*, we observed no statistically significant difference between the transgenic lines and the control. However, bacterial bioassays have shown that transgenic plants presented a significant resistance to *P. syringae* and tolerance to *X. fastidiosa*, both gram-negative phytopathogens. The results from the bioassay conducted with *X. fastidiosa* were particularly reliable as the method used could detect the pathogen with a high degree of sensitivity and specificity, at levels as low as 5–10 cells of the pathogen per reaction.

P. syringae can infect a wide range of crops, such as sugar beet, tomato, wheat, soybean and barley. *X. fastidiosa* is capable of inducing very serious symptoms in the host plant, such as leaf scorch in almonds, variegated chlorosis in citrus (CVC), leaf scorch in coffee, leaf discolouration and scorching in vines (Pierce's disease) and quick decline syndrome in olives (Lindow, 2019). This pathogen is known for its extreme infection capacity, being vectored by many insect species that feed from the xylem of plants; their polyphagy is reflected in the spread of the bacterium to a large number of host species. Tobacco has been used in previous studies as an experimental host in the study of plant–*X. fastidiosa* interactions (Lopes et al., 2000), where it has been possible to discriminate between citrus and coffee *Xylella* isolates by their symptoms (Lopes et al., 2020); thus, tobacco was ideal for our transgenic expression studies.

At present, mechanisms to explain how SMases could act on the antimicrobial/antagonistic interactions with fungi are still not clear. SMases are described as generating ceramide and phosphorylcholine from sphingomyelin, modifying the structure and morphology of the target membranes, leading to cell membrane destabilization (Goni & Alonso, 2002; Jenkins et al., 2009). In addition, bacterial SMases hydrolyse sphingomyelin as well as glycerophospholipids, generating several metabolites that play crucial roles in distinct physiological processes, including membrane dynamics, cellular signalling, migration, growth and death (Flores-Díaz et al., 2016; Li et al., 2019). However, the role of sphingomyelinase activity in inducing resistance to bacterial pathogens is still unknown and further studies must be carried out to establish the antibacterial action of SMases in plants. Nevertheless, sphingomyelin is a lipid that is known to support the growth of *Spiroplasma mirum* in culture medium, a fastidious bacterium as in the case of *X. fastidiosa* (Chang, 1993).

In conclusion, we have demonstrated that the expression of a sphingomyelinase gene from *T. harzianum* induced strong resistance/tolerance to both *P. syringae* and *X. fastidiosa* in the model plant tobacco, which is susceptible to these bacteria. The genetically modified lines generated in this work might be studied further in order to shed light on the mode of action of the ThSMase in triggering

bacterial resistance. Experiments aiming to clarify aspects of protein subcellular localization and transport, secretory activity, mechanisms of signalling pathways related to immune responses, neutralization of bacterial harmful molecules, up- and down-regulation of genes involved in pathogen defence, and inhibition of elicitors of programmed cell death, will help to establish the function and mechanisms of action of SMases in plants.

The strategy presented here could be extended to achieve resistance against other bacterial diseases in distinct crops. This technology is a foundation for the production of bacterial-resistant varieties, resulting in a reduced environmental impact of plant disease management.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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