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A Gene Encoding Scots Pine Antimicrobial Protein Sp-AMP2 (PR-19) Confers Increased Tolerance against *Botrytis cinerea* in Transgenic Tobacco

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Abstract: Both the establishment of sustainable forestry practices and the improvement of commercially grown trees require better understanding of mechanisms used by forest trees to combat microbial pathogens. We investigated the contribution of a gene encoding Scots pine (*Pinus sylvestris* L.) antimicrobial protein Sp-AMP2 (PR-19) to the host defenses to evaluate the potential of *Sp-AMP* genes as molecular markers for resistance breeding. We developed transgenic tobacco plants expressing the *Sp-AMP2* gene. Transgenic plants showed a reduction in the size of lesions caused by the necrotrophic pathogen *Botrytis cinerea*. In order to investigate *Sp-AMP2* gene expression level, four transgenic lines were tested in comparison to control and non-transgenic plants. No Sp-AMP2 transcripts were observed in any of the control and non-transgenic plants tested. The transcript of Sp-AMP2 was abundantly present in all transgenic lines. Sp-AMP2 was induced highly in response to the *B. cinerea* infection at 3 d.p.i. This study provides an insight into the role of Sp-AMP2 and its functional and ecological significance in the regulation of plant–pathogen interactions.

Keywords: antimicrobial protein; plant-fungi interaction; plant defense; disease resistance; heterologous expression; pathogenic fungus

1. Introduction

Plants engage an armory of defenses in response to microbial infection and pathogen attack. Invasion of plant tissues by microorganisms causes large-scale rearrangements in host metabolism, which are achieved via orchestrated changes in expression levels of hundreds of genes. Activation of defense responses is mediated by several types of signaling molecules via specific signaling pathways [1]. The best-characterized examples of such signaling molecules are jasmonic acid (JA) and methyl jasmonate (MeJA), ethylene (ET), and salicylic acid (SA). The activation of corresponding signaling pathways results in the induction of various plant defensive mechanisms, both physical and chemical. Physical defensive mechanisms include, among others, suberization and reinforcement of cell walls, and formation of wound periderm. Accumulation of induced antimicrobial secondary metabolites collectively known as phytoalexins (terpenoids, tannins, stilbenes, alkaloids, etc.), oxidative burst, and induction of antimicrobial and pathogenesis-related (PR) proteins provide examples of plant chemical defenses [2,3]. Pathogen-inducible defense-related proteins, known as PR proteins, have been reported in many plant species to enhance resistance of plants against microbial pathogens

and insect pests [4]. Antimicrobial peptides (AMPs) play a major role in defense, and they are considered to be an important component of the innate immunity in living organisms [5]. Plant AMPs vary in their amino acid composition and structure [6] and are assigned to different families [7]. However, they share fundamental structural properties, such as small size and clustering of the cationic and hydrophobic amino acids within distinct domains [8]. The correlation between the increased production of antimicrobial peptides and improved pathogen resistance has been demonstrated in many commercial species [9,10]. AMPs are considered to be an important tool for the enhancement of the pest resistance in commercial crops, and there are numerous examples where heterologous expression of plant AMPs resulted in increased resistance to pathogens [11]. However, most of the AMPs studied so far originated from angiosperms, and the potential of AMPs from other plant groups (e.g., conifers, ferns, or mosses) remains poorly investigated.

In conifers, pathogenesis-related proteins associated with the defensive responses to *Heterobasidion annosum* (Fr.) Bref. infection include PR-2 (β -1,3-glucanases) [12], PR-3 (chitinases) [13,14], PR-5 (thaumatin-like proteins) [15], PR-9 (class III peroxidases) [16,17], PR-12 (defensin PsDef1) [18], SPI1 [19], and PR-19 (Scots pine antimicrobial proteins, Sp-AMPs) [20,21]. Other PR proteins induced upon pathogen infections and diverse abiotic stress in forest tree species are implicated in the systemic acquired resistance and tree resistance as well (as reviewed elsewhere [22]).

Five genes of Scots pine (*Pinus sylvestris* L.) antimicrobial peptides, Sp-AMPs, induced in response to inoculation with *H. annosum*, have been identified, of which four (*Sp-AMP1–4*) are known to share 93–100% nucleotide sequence identity [20]. Sp-AMPs represent a family of PR proteins, PR-19. PR-19 is proposed to be a family of non-catalytic β -glucan binding proteins that inhibit the hyphal growth of *H. annosum* [21]. Sp-AMP proteins are 105 amino acids-long cysteine-rich proteins, each containing an N-terminal region with a predicted secretion signal peptide. Based on the structure, conserved number and relative spacing of cysteines, Sp-AMP proteins belong to the MiAMP1 (*Macadamia integrifolia* Maiden & Betche AMP) family of proteins [23], which are believed to have a role in the defense against fungal pathogens. The proposed mode of action for these antimicrobial peptides involves interference with glucan assembly and alteration of the fungal cell wall structure, consequently compromising the fungal cell wall integrity [21]. Sp-AMP proteins' applicability for the tree innate immune system is in providing rapid host protection with low energy expense [24].

We explored an effect of the heterologous expression of the Sp-AMP2-encoding gene in the angiosperm model plant tobacco. The main objective of expressing *Sp-AMP2* was to evaluate the potential role of *Sp-AMP* in plant resistance and to elucidate its functional significance in the regulation of phytopathogen-host interaction. The response of the transgenic tobacco expressing *Sp-AMP2* challenged with a fungal pathogen was investigated.

2. Materials and Methods

2.1. *Agrobacterium Tumefaciens* Vectors and Strains

Sp-AMP-encoding genes share a high level of sequence similarity. *Sp-AMP2* has the highest score for identity of the *Sp-AMP* consensus sequence among the other members [20]. The *Sp-AMP2* full-length complementary DNA (cDNA) was obtained by PCR amplification from a subtraction cDNA library (hasp001xd12f; GenBank: BI416519.1) of Scots pine root tissue infected with the root rot fungus *H. annosum*. It was cloned into pT-Adv vector (Clontech, Mountain View, CA, USA) using gene-specific primers containing *Xho*I and *Xba*I restriction sites and twenty-four nucleotides representing the Strep-tag II sequences in the reverse primer (IBA, Göttingen, Germany). The primers were designed based on the coding sequence of *Sp-AMP2* gene (Genebank: AF410953.1) (forward primer: 5' GCACTCGAGATGGAAACCAAGCGCTTGGCAT; reverse primer: 5'-CAGTCTAGATCATTTTTTCGAACTGCGGGTGGCTCCAGCATTGGATGAAAAAACT).

The amplified products were subcloned into the *Xho*I/*Xba*I-digested plant binary vector pE1801 where the *Nco*I site was first deleted by mung bean nuclease trimming and re-ligation. pE1801 (Figure 1)

2.2. Tobacco Transformation

Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was stably transformed using the leaf disc method [28]. Leaves were surface-sterilized for 1 min with 70% ethanol and for 10 min with 1.2% sodium hypochlorite solution, then rinsed five to seven times with sterile water.

The *Agrobacterium* suspension and tobacco leaf discs were incubated in a petri dish for 5 min, then infected leaf discs were placed upside down on agar-solidified MS medium (Sigma-Aldrich, M5524, St Louis, MO, USA) containing 1 mg/L BAP (6-benzylaminopurine, Sigma-Aldrich, M5524, St Louis, MO, USA) and supplemented with sucrose (20 g/L) (Sigma-Aldrich, M5524, St Louis, MO, USA). Plates were kept in the dark for 2 days; thereafter the infected explants were transferred to a fresh solid MS medium containing 1 mg/L BAP and sucrose (20 g/L), and supplemented with 100 mg/L kanamycin and 500 mg/L cefotaxime (Sigma-Aldrich, M5524, St Louis, MO, USA) at 23 °C in the light for 4–6 weeks. Each week, fresh selection plates were changed and, after the emergence of the shoots, a single shoot per leaf disc was transferred to MS Jars containing 100 mg/L kanamycin and 500 mg/L cefotaxime. Transgenic plants were regenerated under kanamycin selection and plantlets were moved into vermiculite in the growth room and then to the green house. Seeds (T1 generation) from the transgenic lines (T0) were collected after self-pollination. For pathogen resistance assay, the wild type plant (control) and four independently transformed T1 lines and their segregating siblings were used.

2.3. Selection of Transgenic Plants

The T1 seeds from transgenic plants were surface-sterilized by immersion in 70% (*v/v*) ethanol for 2 min, followed by immersion in 1.2% sodium hypochlorite solution for 10 min. Afterwards, seeds were washed four times with sterile distilled water and sown onto 1% agar-containing MS medium supplemented with kanamycin (100 mg/L) and sucrose (20 g/L). Plates were incubated at 18 °C with a photoperiod of 16 h. After 14 days, the well-developed rooted seedlings were transferred to soil prepared by mixing peat (Kekkilä Horticulture Peat, Kekkilä Oyj, Finland) and washed sand at a 5:1 ratio (*v/v*). T1 transgenic and non-transgenic plants were generated and sorted based on PCR analysis (XP cycler, BIOER, Hangzhou, China); the PCR product was absent in non-transgenic plants. All plants were grown in a growth room under controlled conditions, were watered when needed, and fertilized weekly with a 1.0% solution of N/P/K (8:4:6 or 16:9:22) fertilizer (Yara, Espoo, Finland).

2.4. cDNA Synthesis by Reverse Transcription

For the initial verification of *Sp-AMP2* in the transgenic plants, RNA was extracted from one T0 transgenic plant, one T1 transgenic plant, one T1 non-transgenic, and one wild type control plant according to the methods of Chang et al. [29]. RNA concentration and quality were assessed spectrophotometrically using a NanodropTM ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). A 1 µg sample of each total RNA isolate was used for cDNA synthesis, as described by Jaber et al. [30]. To verify the transcript of *Sp-AMP2* in the tested tobacco plants, the resulting cDNA was diluted (1:5) and 5 µL was used to amplify the *Sp-AMP2* fragment. The PCR products were separated on a 1.5% agarose gel.

2.5. Pathogen Bioassays of Transgenic Plants

Botrytis cinerea Pers. isolate B05.10 [31], a haploid aggressive strain isolated from *Vitis vinifera* L. (kindly provided by Mehmet Ali Keçeli, Department of Biosciences, Faculty of Biological and Environmental Sciences, University of Helsinki) was used for the pathogen assay.

B. cinerea was grown on potato-carrot agar (PCA). PCA was prepared as follows: 300 g of diced potatoes and 25 g of diced carrots were boiled in water for 30 min, then mashed and filtered through Miracloth (Millipore, Billerica, MA, USA). The final volume was adjusted to 1 L with H₂O, and 15 g of agar was added. *B. cinerea* was grown for 10 days in the dark at approximately 22 °C. Spores were harvested from each dish by rinsing with 10 mL PDB (Potato Dextrose Broth, Difco, Detroit, MI, USA)

while agitating the colonies gently with a glass rod. The spores in PDB medium were vortexed for 10 min at maximum speed and then filtered through Miracloth. The concentration of *B. cinerea* spores was adjusted to 1×10^6 spores/mL. Four-week-old T1 generation tobacco plants and non-transformed control plants were inoculated with 10 μ L of the spore suspension of *B. cinerea* onto the upper surface of a leaf of the growing seedling. Wild type plants (control) and four T1 lines were tested; five plants per line including four transgenic T1 plants and one non-transgenic T1 plant were used for each treatment and evaluated for disease development. To determine the susceptibility of plants, disease lesions were scored at 3 and 5 days post-inoculation (d.p.i.) and recorded using an optical Olympus CX31 microscope equipped with a digital Olympus SC30 camera (Olympus, Tokyo, Japan).

2.6. PCR Analysis of Transgenic Plants

Genomic DNA was isolated using a CTAB (cetyl trimethylammonium bromide) method [32]. The genomic DNA samples of the control plants and transformed T0 lines were analyzed by PCR (XP cycler, BIOER, Hangzhou, China) with the same primers that were used for cloning. The size of the amplified product matched the predicted length of 358 bp. Amplification was performed under the following conditions: 95 °C for 5 min, 39 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 40 s. Finally, the PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Segregating T1 lines were tested using PCR to verify segregating transgenic plants.

2.7. Real-Time Quantitative PCR Analysis

The analysis of the expression levels of the *Sp-AMP2* and marker genes for the SA/JA/ET signaling pathways was performed by real-time quantitative reverse transcription PCR (qRT-PCR, LightCycler[®] 480 II system, Roche Diagnostics, Basel, Switzerland). For this purpose, RNA was extracted from transgenic, non-transgenic, and control plants. For each of the mentioned plant lines, 100 mg of non-treated young leaves (control), leaves inoculated with 10 μ L of PDB (mock inoculation), and leaves inoculated with 10 μ L of the spore suspension of *B. cinerea* (treatment) were taken for RNA isolation. cDNA synthesis by reverse transcription was performed according to the protocol described above.

qRT-PCR was performed with the LightCycler[®] 480 II system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The list of gene names, accession numbers, qRT-PCR primers, and product sizes is provided in Supplementary file S1. PCR thermal conditions consisted of initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 1 min at the appropriate annealing temperature. The specificity of the primer pairs was verified both by gel and by melting curve analysis. The transcript abundance was estimated with LightCycler software version 3.5 (Roche) using SYBR[®] Green PCR Master Mix (Invitrogen) according to the manufacturer's recommendations, based on three biological and three technical replicates for each gene. The gene for elongation factor 1a (*EF-1a*), a very stably expressed gene [33], was used as a reference gene. In agreement with the previous reports, the expression level of this gene showed very little variation in our experiments (mean cycle threshold (Ct) value $Ct \pm SE = 26.91 \pm 0.03$, $n = 324$, where SE indicates standard error). The raw Ct values of the tested genes were normalized to the reference gene and used to compare the results from untreated control wild type against all samples using the $2^{-\Delta\Delta Ct}$ method, as described previously [34].

2.8. Statistical Analyses

All experiments were repeated three times, and similar results were obtained. Means and standard errors were estimated from three independent experiments. Error bars indicate the standard error of the mean across three biological replicates. Statistical significance analyses of the necrosis were performed using paired *t*-test as well as by two-way ANOVA test. Bonferroni post hoc test was applied on the data to compare the value of each column factor "necrosis length" and each row factor "Genotype". $p > 0.05$ was considered to be non-significant (Supplementary file S2). Statistical significance of the pathogen assays on transgenic plants and the gene expression were calculated by

two-way ANOVA test. Thereafter, Bonferroni post hoc test correction was applied on the data for comparisons. The threshold for fold changes in the expression of the differentially expressed genes in comparison to the control samples was set at 2-fold. Asterisks indicate the significance of differences, * p values < 0.05, ** p values < 0.01, and *** p values < 0.001.

3. Results

3.1. Generation of *Sp-AMP2* Transgenic Tobacco Plants

The Scots pine *Sp-AMP2* cDNA under control of a strong promoter was introduced into tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) by *Agrobacterium tumefaciens*-mediated transformation method (Figure 1). The promoter in the vector is a chimeric promoter derived from the octopine and mannopine synthase genes that is approximately 156-fold stronger than the cauliflower mosaic virus (CaMV) 35S promoter in tobacco leaf tissue, which makes it useful for high level constitutive expression of genes [25]. Presence of the *Sp-AMP2* gene in the genomic DNA of tobacco plants was confirmed by PCR amplification. A 358 bp DNA fragment was detected in T0 transgenic tobacco lines, whereas no bands were detected in the untransformed control tobacco plant (Figure 2a). Four different tested tobacco lines T0 were selected for T1 seed collection after self-pollination, and used for further analysis. T1 seeds were collected and verified for transgene presence in plants regenerated on selective medium by PCR (Figure 2b). Transcription of *Sp-AMP2* was confirmed by PCR amplification of *SpAMP2*-specific fragments from the cDNA of the non-transformed wild type, T0, non-transgenic and transgenic T1 plants (Figure 3). The T0 and T1 transgenic lines showed the transcript abundance of *Sp-AMP2*.

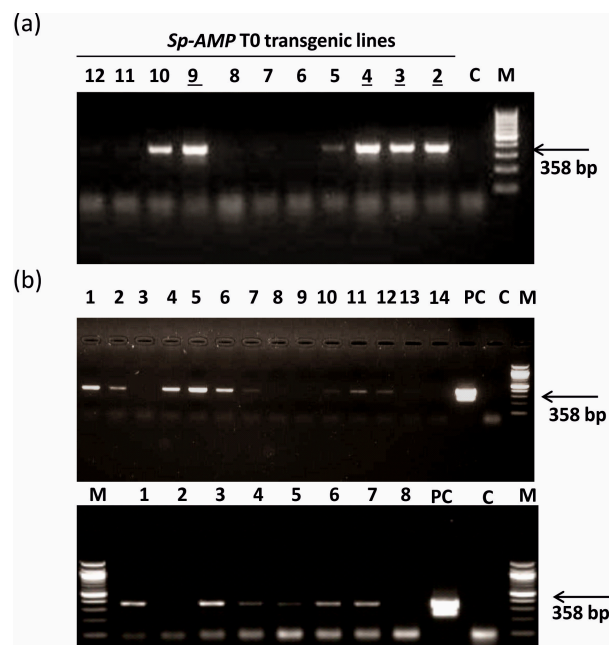


Figure 2. PCR analysis of DNA isolated from transgenic and control tobacco lines. (a) Fragment of 358 bp was generated from T0 plants using *Sp-AMP2*-specific primers. Four lines were selected to generate T1 lines (underlined). Lanes 2–12: T0 transgenic tobacco lines; lane C: non-transformed tobacco (control); lane M: 100 bp DNA ladder. (b) Fragment of 358 bp was generated from T1 plant using *Sp-AMP2*-specific primers. Upper gel: lane 1: segregating T1 line 4 tobacco plant; lanes 2–7: segregating T1 line 3 tobacco plants; lanes 8–13: segregating T1 line 2 tobacco plants; lane 14: no template DNA (water); lane PC: plasmid pE1801-*Sp-AMP* (positive control); lane C: non-transformed tobacco (negative control); lane M: 100 bp DNA ladder. Lower gel: lane M: 100 bp DNA ladder; lanes 1–7: segregating T1 line 9 tobacco plants; lane 8: no template DNA (water); lane PC: plasmid pE1801-*Sp-AMP* (positive control); lane C: non-transformed tobacco (control); lane M: 100 bp DNA ladder.

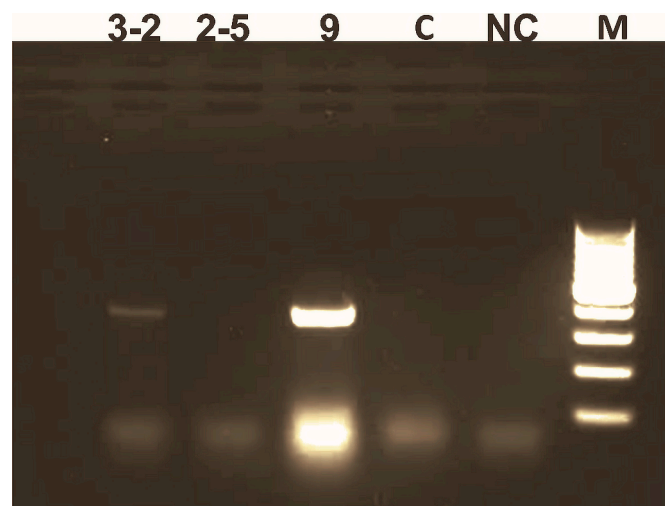


Figure 3. Analysis of the *Sp-AMP2* gene expression in the transgenic tobacco plants by amplification of the gene-specific product from cDNA. Lane 3–2: transgenic T1 plants; lane 2–5: non-transgenic T1 plants; lane 9: T0 plants; lane C: control non-transgenic plants; lane NC: PCR without DNA template; lane M: 100 bp ladder.

3.2. *Sp-AMP2*-Transformed Tobacco Plants Show Increased Tolerance to *B. cinerea*

The effects of infection caused by *B. cinerea* were investigated on the T1 transgenic plants and compared with both non-transformed plants and their non-transgenic segregating siblings. The T1 plants were verified by rooting in selective media and PCR. Each transformation event comprising of three transgenic T1 plants was compared to two non-transgenic T1 sibling plants and to one non-transformed control plant. The sizes of the lesions determined from 3 to 5 days post-inoculation (d.p.i.) under conditions described in the material section are indicated in Figure 4. Necrotic lesions caused by *B. cinerea* on the control tobacco and non-transgenic T1 leaves were more severe and larger than those formed on the leaves of T1 transgenic lines. The lesions on the transgenic lines usually increased slightly after 2 extra days of incubation, however, none reached the same size as those in the non-transformed control. Transgenic tobacco showed significant difference in lesion size in response to infection by *B. cinerea* as compared to the non-transgenic in the two time points (Supplementary file S3). Similar levels of lesion development were observed on non-transgenic T1 plants, compared to those of control plants. However, the disease spread was slower on transgenic plants (Figure 4). No statistically significant differences in the size of necrotic lesions were observed among different transgenic lines expressing the *Sp-AMP2* gene.

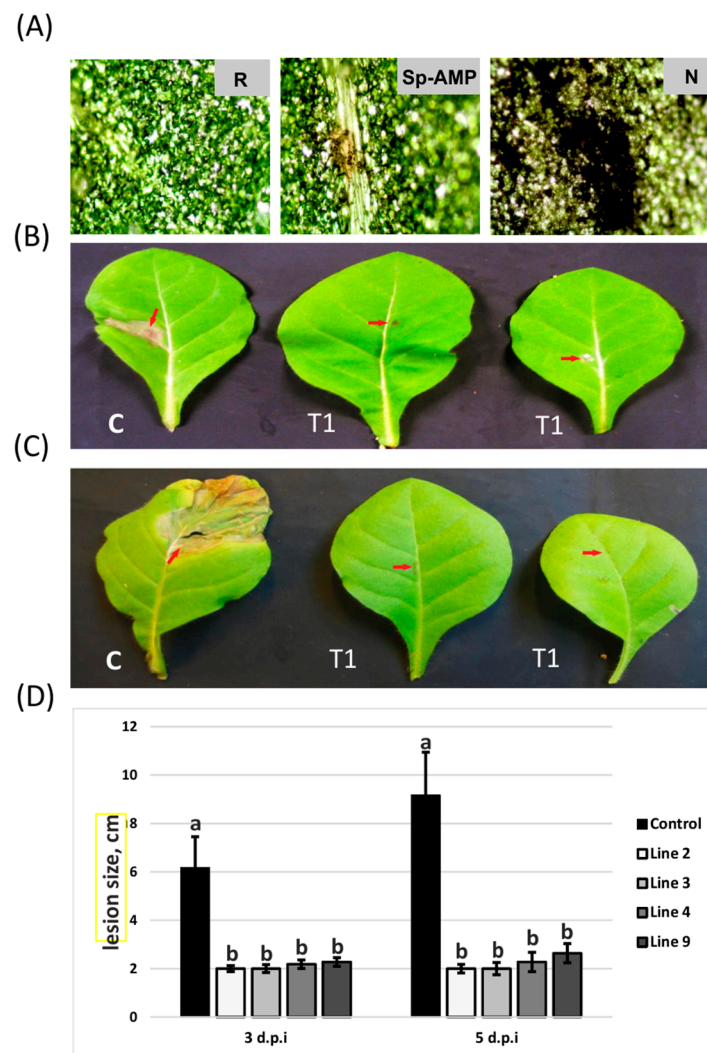


Figure 4. Disease evaluation in *Sp-AMP2*-transgenic tobacco plants. (A) Left to right: leaf surface of non-transgenic tobacco with no infection (R); spores inoculation symptoms caused by *B. cinerea* on a leaf surface of transgenic tobacco 3 days post-inoculation (Sp-AMP); spores inoculation symptoms caused by *B. cinerea* on a leaf surface of non-transgenic tobacco 3 days post-inoculation (N). (B) Size of lesions (indicated with red arrows) formed in leaves 3 days after inoculation with *B. cinerea* on wild type (C) and transgenic plants (T1). (C) Size of lesions progressed in leaves 5 days after inoculation with *B. cinerea* on the same plants. (D) Analysis of lesion sizes on transgenic lines T1 leaves overexpressing *Sp-AMP2* inoculated with *B. cinerea* compared to non-transgenic T1 and wild type plants (control). Error bars indicate the standard error of the mean across three biological replicates. Letters indicate statistically significantly different groups identified by ANOVA analysis ($p < 0.001$).

3.3. Expression Profiles of *Sp-AMP2* Gene and SA/JA/ET Gene Markers in Transgenic Plants

In order to investigate *Sp-AMP2* gene expression level, four transgenic lines were tested in comparison to control and non-transgenic plants. No *Sp-AMP2* transcripts were observed in any of the control and non-transgenic plants tested. The transcript of *Sp-AMP2* was abundantly present in all transgenic lines. *Sp-AMP2* was induced highly in response to the *B. cinerea* infection at 3 d.p.i. In the three transgenic lines (except for line 4) and the expression was sustained over time by the pathogen (5 d.p.i) (Figure 5).

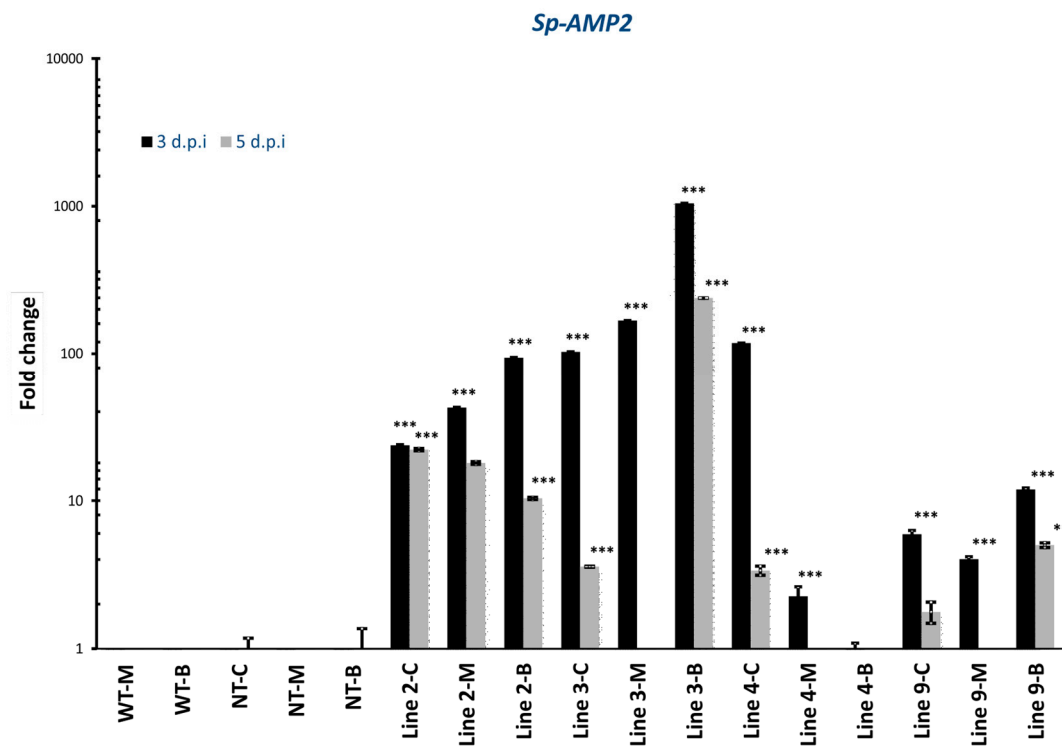


Figure 5. Quantitative real-time PCR analysis of expression patterns of *Sp-AMP2* in different tissues and in response to various treatments at 3 and 5 days post-inoculation. *Sp-AMP2* relative expression levels were determined as described earlier (Livak and Schmittgen 2001 [34]) and normalized with Elongation factor 1a (*EF-1a*). WT-M, untransformed wild-type tobacco mock treated with PDB (Potato Dextrose Broth); WT-B, untransformed wild-type tobacco infected with *B. cinerea*; NT-C, non-transgenic tobacco not treated control; NT-M, non-transgenic tobacco mock treated with PDB; NT-B, non-transgenic tobacco with independent transgenic tobacco infected with *B. cinerea*; Line 2; Line 3; Line 4; Line 9, independent transgenic line expressing *Sp-AMP2* with either non-treated control (C), infected with *B. cinerea* (B), or mock treated with PDB (M). Data represent the fold change of *Sp-AMP* compared with expression in untreated wild type control plants at the corresponding time points. Error bars indicate the standard error of the mean across three independent biological replicates. Asterisks indicate the level of significance in comparison to non-transgenic plants infected with *B. cinerea* (NT-B), *** p values < 0.001.

To study the interaction between the SA and JA/ET signaling pathways and the expression of *Sp-AMP2* in the transgenic tobacco, we investigated the expressions levels of two important marker genes. *PR1a* is a SA-associated gene that serves as a common indicator for SA signaling pathway activity [35]. *ERF1* (*ethylene response factor 1*) is a marker gene, which is induced by simultaneous action of the JA and ET signaling pathways [36]. The induction of *ERF1* was observed in all tested tobacco plants at 3 d.p.i. However, increased levels of *ERF1* transcripts were observed in the transgenic plants, notably in two lines challenged with *B. cinerea* (Lines 3 and 9) (Figure 6). *PR1a* was strongly induced in the mock-inoculated wild type and in two transgenic lines (Lines 4, 9). The highest expression was observed in the transgenic line 9 (Figure 6).

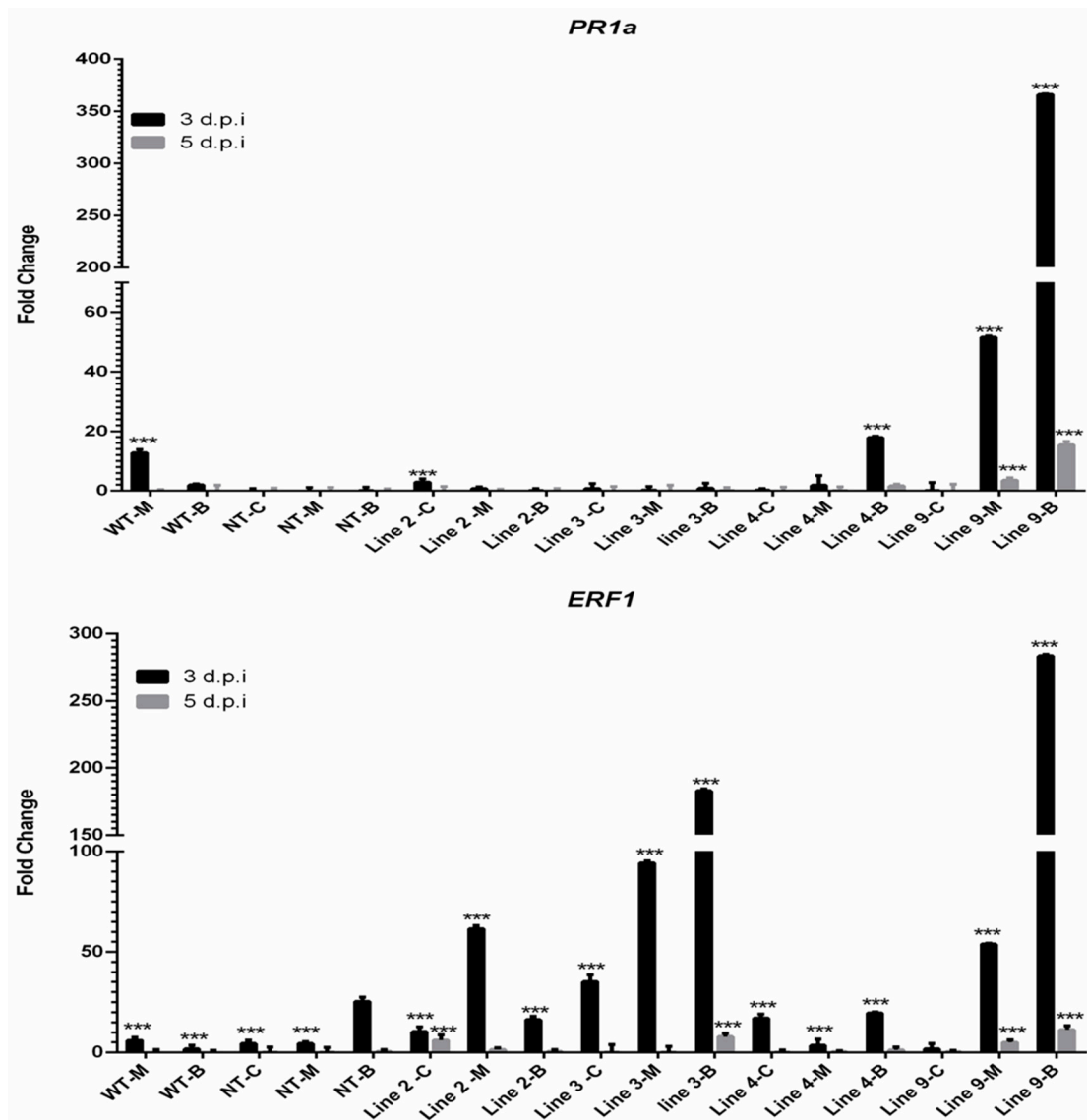


Figure 6. Transcript abundance of tobacco *PR1a* (above) and *ERF1* (below) genes in different tobacco plants at 3 and 5 days post-inoculation with either non-treated control (C), either infected with *B. cinerea* (B), or mock treated with PDB (M). *PR1a* and *ERF1* relative expression levels were determined as described earlier (Livak and Schmittgen 2001 [34]) and normalized with Elongation factor 1a (*EF-1a*). WT-M, untransformed wild-type tobacco mock treated with PDB; WT-B, untransformed wild-type tobacco infected with *B. cinerea*; NT-C, non-transgenic tobacco non-treated control; NT-M, non-transgenic tobacco mock treated with PDB; NT-B, non-transgenic tobacco with independent transgenic tobacco infected with *B. cinerea*; Line 2; Line 3; Line 4; Line 9, independent transgenic line expressing *Sp-AMP2* with either non-treated control (C), infected with *B. cinerea* (B) or mock-treated with PDB (M). Data represent the fold change of *Sp-AMP* compared with expression in untreated wild type control plants at the corresponding time points. Error bars indicate the standard error of the mean across three independent biological replicates. Asterisks indicate the level of significance in comparison to non-transgenic plants infected with *B. cinerea* (NT-B), *** *p* values < 0.001.

4. Discussion

In the present study, we investigated the role of a gene encoding Scots pine antimicrobial peptide *Sp-AMP2* in the defense against pathogenic fungi. Our earlier results showed strong *Sp-AMP* induction in Scots pines challenged with the necrotrophic fungal pathogen *H. annosum* [20,21], justifying further

detailed functional analyses of the corresponding gene. A successful transfer of a Sp-AMP2-encoding gene of the gymnosperm (Scots pine) to the angiosperm recipient (tobacco) was demonstrated. No homologs of *Sp-AMP* genes have been identified either in *Nicotiana benthamiana* Domin or in *Nicotiana tabacum* [37]. Our results suggest that expression of *Sp-AMP2* in transgenic tobacco conferred enhanced resistance to *Botrytis cinerea*. This is a further functional demonstration of the potential role of Sp-AMP2 in plant defense. The plants expressing *Sp-AMP2* showed a reduction in the spread and subsequent expansion of fungal infection over the 5 day evaluation period, with the most significant statistical differences being observed at 5 d.p.i., indicating that Sp-AMP2 is actively involved in the inhibition of *B. cinerea* and suggesting a broader spectrum of the Sp-AMP2 inhibitory activity. The observed delay in symptom development could potentially allow plants either to recover or adapt to changes in field conditions that could potentially reduce disease spread.

A considerable variation in the expression level of the heterologous *Sp-AMP2* gene among different lines of transgenic tobacco plants was observed in our experiments. The reasons for these differences are not entirely clear, as the gene was put under control of a strong constitutive promoter. One of the possible explanations could be the integration of the transgenic construct in different genomic loci of the transgenic host plants, which in turn might have an effect on the expression level of the transgene. However, the transgenic lines with different expression levels of the *Sp-AMP2* gene showed no statistically significant differences in the size of necrotic lesions caused by inoculation with *B. cinerea*. It is likely that even low-level expression of *Sp-AMP2* is sufficient to protect transgenic tobacco plants from this fungal pathogen.

Tobacco plants expressing *Sp-AMP2* showed increased level of *ERF1* induction upon pathogen challenge, indicating a potential interplay between *Sp-AMP* expression and JA/ET signaling pathways. In Scots pine, increased expression of *Sp-AMP* in response to pathogen challenge, salicylic acid, ethylene, and other environmental stresses was shown in our earlier study [21]. SA-dependent signaling pathway, which is engaged with the cell death machinery against biotrophic pathogens, controls the synthesis of low molecular weight antimicrobial proteins such as PR-1, PR-2, and PR-5. At the same time, the JA/ET-dependent pathway is associated with defense against necrotrophic pathogens, and it activates genes that encode antimicrobial proteins such as thionin (THI2.1), defensin (PDF1.2), PR-3, and PR-4 [38]. Such differences may affect the specificity of plant-pathogen interactions.

PR-19 family members are potential alternatives to current *H. annosum* control and management strategies, since they exploit built-in defense systems of the host trees. A long-term objective is to find a gene that is tightly linked to a disease resistance trait that would serve as molecular DNA marker to be applied in breeding programs and facilitate the selection of naturally occurring genotypes with higher resistance against the root rot pathogen.

A similar cysteine-rich peptide, MiAMP1 protein, has been shown to be highly inhibitory to a wide range of phytopathogens, particularly fungi. In addition, transgenic expression of *MiAMP1* in canola (*Brassica napus* L.) provided enhanced resistance against blackleg disease caused by the fungus *Leptosphaeria maculans* Ces. & De Not [39]. Also, another MiAMP1-related cysteine-rich antimicrobial peptide from western white pine (*Pinus monticola* Douglas ex D. Don) (*PmAMP1*) that belongs to the same family of MiAMP1, and is the closest homolog to Sp-AMPs, has been successfully transformed into canola and proved to confer resistance to multiple fungal pathogens (*Alternaria brassicae* (Berk.) Sacc., *L. maculans*, and *Sclerotinia sclerotiorum* (Lib.) de Bary) [40]. Further genetic association studies on western white pine *PmAMP1* in a collection of western white pine trees revealed that high quantitative disease resistance against white pine blister rust (WPBR) caused by the obligate biotrophic fungus *Cronartium ribicola* A. Dietr. is genetically attributed (albeit slightly) to multiple *PmAMP1* haplotypes, suggesting that functional regulation of many other defense-related genes is contributing to the quantitative variations of disease resistance. However, the study identified a haplotype of *PmAMP1* (*PmAMP1*-hap2) which confers a higher resistance to *C. ribicola* than other haplotypes and which would therefore certainly benefit traditional tree breeding by marker-assisted selection [41].

The cysteine-rich antimicrobial peptides are evolutionarily dynamic within plant genomes, which are highly adaptive in the challenge of more evolutionary flexible pathogen populations [42]. Moreover, the structural resemblance between MiAMP1 protein family members and the novel Sp-AMP does not necessarily reflect functional similarities [43]. Therefore, the functional validation of the novel Sp-AMPs from the Scots pine through this transgenic experiment is imperative to manifest its potential in plant disease immunity.

Other authors have also demonstrated that somatic embryos of Norway spruce (*Picea abies* (L.) H. Karst.) plants at an age of 8 to 10 months over-expressing the SPI1 spruce defensin protein displayed reduced fungal growth in the sapwood after inoculation with *H. annosum* [44]. Also, SPI1 spruce defensin that was expressed in tobacco permitted less growth of the bacterial pathogen *Erwinia carotovora* (Jones) Bergey et al. [45], suggesting that SPI1 increases resistance in both homologous and heterologous systems. Furthermore, poplar trees transformed with a gene encoding synthetic peptide D4E1 were found to be resistant to bacterial pathogens *Agrobacterium tumefaciens* and *Xanthomonas populi* (Rid ) van den Mooter & Swings but not to the fungal pathogen *Hypoxyylon mammatum* (Wahlenb.) P. Karst [46].

The combination of the broad spectrum action of MiAMP1 protein family members on numerous microorganisms [47,48] and the nature of the cysteine-rich AMPs, which reduce the growth of major microbes without any toxic effects toward the host [49], makes Sp-AMP proteins potential candidates for developing pathogen-resistant crops, a further demonstration of their role in tree resistance.

5. Conclusions

In this study, in order to address the role of Sp-AMPs in plant defense, *Sp-AMP2* was inserted into the genome of the tobacco model plant. The transformed tobacco plants with *Sp-AMP2* showed increased tolerance against *Botrytis cinerea*. Our future study will explore the possibility to transfer the *Sp-AMP2* into a related conifer tree species using most promising technology approaches [50] and further assess its role in tree resistance.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/9/1/10/s1, Supplementary file S1: List of primers used for the real-time quantitative PCR assays, Supplementary file S2: Statistical significance analyses of the genes expression, Supplementary file S3: Statistical significance analyses of the lesion size.

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