A patatin-like protein synergistically regulated by jasmonate and ethylene signaling pathways plays a negative role in *Nicotiana attenuata* resistance to *Alternaria alternata*

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**A B S T R A C T**

Although patatin was initially identified as a major storage protein in potato tubers, patatin-like proteins (PLPs) have been recently reported to be widely present in many plant species and shown to be involved in plant-pathogen interactions. However, it is not clear whether PLPs are involved in *Nicotiana attenuata* resistance against the necrotrophic fungal pathogen, *Alternaria alternata*. In this study, we identified a NaPLP gene, whose expression was highly elicited by *A. alternata* inoculation. Silencing NaPLP enhanced *N. attenuata* resistance to *A. alternata*, which was associated with higher induction levels of JA and ethylene biosynthetic genes, *NaACS1*, *NaACO1* and *NaLOX3*. The induction of NaPLP expression by the fungus was abolished in JA-deficient plants and significantly reduced in ethylene-insensitive plants. In addition, NaPLP transcripts were highly induced by exogenous treatment with either methyl jasmonate (MeJA) or ethylene. Co-treatment with MeJA and ethylene led to a much higher induction level of NaPLP transcripts, and this synergistic induction was largely dependent on endogenous JA and ethylene signaling pathways. Thus, we conclude that the NaPLP gene is elicited by *A. alternata* via JA and ethylene signaling pathways in a synergistic way; however, unlike other JA- and ethylene-induced defense genes, NaPLP negatively affects plant resistance to the fungus likely by suppressing JA and ethylene biosynthetic gene expression.

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1. **Introduction**

Early stages of pathogen infection in plants are usually associated with the production of phytohormones. Although there are some exceptions, jasmonate (JA) and ethylene signaling pathways are usually induced for necrotrophic pathogen resistance, while the salicylic acid pathway for resistance against biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). In response to *Alternaria alternata*, a notorious necrotrophic fungal pathogen causing brown spot disease in leaves of *Nicotiana* species (Sun et al., 2014a), wild tobacco *Nicotiana attenuata* plants activate both jasmonate (JA) and ethylene signaling pathways to defend against this pathogen (Sun et al., 2014b, 2017; Li and Wu, 2016). Plants impaired in either JA (or ethylene) production or perception are highly susceptible to the fungus (Sun et al., 2014b, 2017), supporting the idea that host plants activate JA and ethylene signaling pathways for resistance against necrotrophic pathogens.

Patatin is a glycoprotein which contributes about 40% of the total soluble protein in potato tubers (Racusen, 1983). The protein has a conserved esterase motif (Gly-X-Ser-X-Gly) and has been shown to have lipid acyl hydrolase activity, catalyzing the nonspecific hydrolysis of phospholipids, glycolipids, sulfolipids and mono- and diacylglycerols (Rydel et al., 2003). Recently, it has been found that patatin-like proteins (PLPs) are present in many plant species and have been reported to be involved in plant-pathogen interactions. In *Arabidopsis*, AtPLP2 expression is strongly induced in leaves after *Botrytis cinerea* infection (La Camera et al., 2005). Furthermore, plants silenced for AtPLP2 show increased resistance to *B. cinerea*, whereas plants overexpressing AtPLP2 are more susceptible to this necrotrophic fungus (La Camera et al., 2005), indicating that AtPLP2 plays a negative role in resistance against *B. cinerea* by facilitating fungal colonization.

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In this study, an *A. alternata*-elicited *NaPLP* was identified. The role of *NaPLP* in resistance against *A. alternata* and transcriptional regulation of JA, ethylene, and phytoalexin scopoletin biosynthetic genes were investigated in *NaPLP*-silenced plants through a virus-induced gene silencing (VIGS) technique. In addition, the transcriptional regulations of this gene by *A. alternata*, JA and, ethylene were investigated in WT and plants impaired in JA or ethylene signaling pathways.

2. Materials and methods

2.1. Plant and fungal material

Seeds of the 31st generation of an inbred line of *N. attenuata* were used as the wild type (WT) genotype. Stably transformed lines of ethylene-deficient (irACO), ethylene-insensitive (Ov-etr1), JA-deficient (irAOC), and JA-insensitive (irCO1) plants have been previously generated (von Dahl et al., 2007; Kallenbach et al., 2012; Krügel et al. (2002).

2.2. Generation of VIGS plants

The specific 387-bp PCR fragment used for silencing *NaPLP* (XM_019396434) was amplified by the primer pair (CJ07: 5ʹ-CAT-AAGCTTCCCTTCTCTGCTGCCAAAG-3ʹ and CJ08: 5ʹ-CATGGATCCGCACGACACACCATC-3ʹ), and subsequently cloned into pTV00. To generate *NaPLP*-silenced plants (VIGS *NaPLP*), Agrobacterium tumefaciens (CV3101) cells carrying the above construct were combined with cells having pBINTRA, and were inoculated into leaves of *N. attenuata*. Green tissue of *N. attenuata* were grown and used for inoculation as described in Sun et al. (2014a).

3.1. A. alternata infection elicits *NaPLP* expression

Transcriptome analysis of *N. attenuata* source-sink transition leaves (0 leaves) inoculated with *A. alternata* revealed that a Potatin-like protein gene (*NaPLP*; Genebank accession number: XM_019396434) was highly elicited one day post inoculation (dpi). Sequence comparisons and alignments showed that this *NaPLP* protein has 65% sequence similarity to the potato tuber patatin and shares a strictly conserved serine hydrolase/lipase motif CXSXG (Fig. 1). To confirm the up-regulation of *NaPLP* expression after inoculation, its transcriptional levels were quantified by real-time PCR analysis at 1 and 3 dpi. Compared to mock controls, *NaPLP* transcripts increased to 16.8-fold at 1 dpi, and reached to 40.8-fold at 3 dpi (Fig. 2).

3.2. Silencing *NaPLP* enhances plant resistance to *A. alternata* and transcription of *NaACS1*, *NaACO1* and *NaLOX3*

To study the role of *NaPLP* in *N. attenuata* resistance to *A. alternata*, we silenced *NaPLP* via virus-induced gene silencing (VIGS). Compared with mock controls, transcripts of *NaPLP* were dramatically induced in young leaves of *N. attenuata* plants transformed with empty vector (EV) at 3 dpi; however, plants transformed with the *NaPLP*-silencing construct (VIGS *NaPLP*) showed a 92% reduction in *NaPLP* transcripts compared to EV plants with the same treatments, indicating effective silencing of the *NaPLP* (Fig. 3a).

We measured the necrotic lesion diameter at 5 dpi in 0 leaves of *VIGS* EV and EV plants to investigate whether *NaPLP*-silencing affects *N. attenuata* resistance to *A. alternata*. The lesion diameter in...
two independent VIGS experiments decreased significantly; the average diameter of lesions in EV plants was 6.14 ± 0.15 cm, whereas in VIGS NaPLP plants the average diameter was 4.56 ± 0.23 cm (Fig. 3b). This result indicates that NaPLP is a negative regulator of plant resistance to A. alternata.

To further understand the role of NaPLP in plant resistance, we investigated the transcriptional levels of crucial genes for JA (NaAOS, NaLOX3), ethylene (NaACS1, NaACO1) and scopoletin (NaF6'H1) biosynthesis in VIGS NaPLP plants. Compared to EV plants, VIGS NaPLP plants had significantly higher transcriptional levels of NaACS1, NaACO1, and NaLOX3 (Fig. 4a, b, d); in contrast, NaAOS and NaF6'H1 were elicited to the same levels in EV and VIGS

![Fig. 1. Comparison of patatin-like protein sequences. The amino acid sequence of NaPLP (Nicotiana attenuata, XP_019251979.1) was compared with AtPLP2 (Arabidopsis thaliana, AAM13304.1) and patatin (Solanum tuberosum, CA25592.1). Consensus sequences are shaded with black and the serine hydrolase GXSXG motif is indicated in red font (bracket).](image1)

![Fig. 2. Elicitation of NaPLP by A. alternata in WT plants. NaPLP transcripts were measured by real-time PCR in the source-sink transition leaves (0 leaves) of WT plants treated with mock or with A. alternata at 1 and 3 days post inoculation (dpi). All transcriptional levels were normalized with a housekeeping gene NaActin II. Values are means ± SE for five biological replicates. Asterisks indicate the level of significant difference between WT and transgenic plants with the same treatments (Student’s t-test: ***, p < 0.005).](image2)

![Fig. 3. Silencing NaPLP enhances plant resistance to A. alternata. a) Mean ±SE NaPLP transcript levels were measured by real-time PCR in five biological replicates of young leaves of EV and VIGS NaPLP plants at 3 dpi. b) Mean ±SE diameter of necrotic lesions of fifteen replicates of young leaves of EV and VIGS NaPLP plants at 6 dpi. Asterisks indicate the level of significant difference between EV and VIGS NaPLP plants (Student’s t-test: *, p < 0.05; ***, p < 0.005).](image3)
NaPLP plants (Fig. 4c, e). These results indicate that biosynthesis of JA and ethylene is enhanced in VIGS NaPLP plants, whereas scopoletin-based defense is not affected.

3.3. Regulation of NaPLP gene expression by JA and ethylene signaling pathways

To test whether the elicitation of NaPLP expression by A. alternata is dependent on JA and ethylene signaling pathways, we investigated the expression of NaPLP in JA-deficient (irAOC) and ethylene-insensitive (Ov-etr1) plants. The induction of NaPLP by A. alternata was completely abolished in irAOC plants at both 1 and 3 dpi (Fig. 5a), indicating that the elicitation of NaPLP expression is completely JA dependent. Ov-etr1 plants accumulated 75% of the NaPLP transcripts that WT had at 1 dpi, and only 26.4% at 3 dpi (Fig. 5a), suggesting that ethylene signaling is also required.

Because A. alternata-induced NaPLP expression was dependent on JA and ethylene signaling pathways, we reasoned that the transcriptional levels of NaPLP could be elicited by exogenous JA or ethylene treatments. NaPLP transcripts increased 12.4-fold one hour after MeJA treatment, and 457-fold and 474-fold at 3 and 6 h after treatment, respectively (Fig. 5b). Ethephon, a synthetic compound which decomposes into ethylene upon metabolism by plants, also increased NaPLP expression from 1.69-fold at 1 h, to 24.55-fold at 3 h, and 2619-fold at 6 h after treatment (Fig. 5b). Interestingly, co-treatment with MeJA and ethephon dramatically increased transcriptional levels of NaPLP from 52.25-, 5457.13-, and 33129.40-fold after 1, 3 and 6 h treatments, respectively (Fig. 5b). These results indicate that NaPLP expression can be induced by JA and ethylene signaling both individually and synergistically.

3.4. Synergistic induction of NaPLP is dependent on endogenous JA and ethylene signaling pathways

To test whether the synergistic induction of NaPLP by MeJA and ethephon was dependent on endogenous JA and ethylene signaling pathways, we treated the 0 leaves of WT, irAOC (JA-deficient), irCOI1 (JA-insensitive), irACO (ethylene-deficient), Ov-etr1 (ethylene-insensitive) plants with MeJA and ethephon simultaneously. The results revealed that NaPLP was synergistically induced in WT and irAOC plants at both 24 and 72 h, but not in irCOI1 plants after the same elicitation (Fig. 6a). Similarly, NaPLP was synergistically induced in WT and irACO plants but not in Ov-etr1 plants (Fig. 6b). These results indicate that both JA and ethylene perception are required for the synergistic induction of NaPLP by MeJA and ethephon.
JA and ethylene signaling pathways play central roles in plant defense against necrotrophic pathogens. In *N. attenuata*, they regulate a series of defense-related genes, including *NaFSH1*, the gene encoding the key enzyme for phytoalexin scopoletin and scopolin biosynthesis (Sun et al., 2014b; Li and Wu, 2016); and *NaPDR1* and *NaPDR1*-like, two pleiotropic drug resistance transporter genes required for resistance (Xu et al., 2018). *NaPLP* was highly elicited after *A. alternata* inoculation, and this induction was clearly dependent on JA and ethylene signaling pathways (Figs. 2 and 5). Indeed, exogenous treatments with either MeJA or ethephon induced *NaPLP* expression (Fig. 5). Interestingly, *NaPLP* transcripts were synergistically induced by MeJA and ethephon co-treatments, and this elicitation was completely dependent on endogenous JA and ethylene signaling pathways (Figs. 5 and 6). All these data indicate that the elicitation of *NaPLP* expression by *A. alternata* likely results from the synergistic induction of *NaPLP* by endogenous JA and ethylene signaling pathways. However, unlike other JA- and ethylene-regulated defense genes, silencing *NaPLP* increased plant resistance. Thus, this study provides a good example of a JA- and ethylene-regulated gene playing a negative role in pathogen resistance.

Taken together, our results indicate that we have identified an *A. alternata*-induced *NaPLP* gene, whose elicitation by the fungus likely results from JA and ethylene signaling pathways in a synergistic way, and unlike other JA- and ethylene-induced defense genes, it negatively affects plant resistance to the fungus possibly through suppression of JA and ethylene biosynthesis.

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4. Discussion

Although patatin was initially identified as a major storage protein, more and more reports indicate that patatin-like proteins exist widely in many plant species and play a role in plant-pathogen interactions; for example, AtPLP2 in *Arabidopsis* confers susceptibility to necrotrophic fungus *B. cinerea* (La Camera et al., 2005). In this study, we also found that NaPLP is a susceptible factor in the *N. attenuata* – *A. alternata* pathosystem. Silencing *NaPLP* in *N. attenuata* plants increased the resistance to the pathogen *A. alternata* (Fig. 3). How NaPLP negatively regulates *N. attenuata* resistance to *A. alternata* remains unclear. We found that NaACS1, NaACO1 and NaLOX3 were elicited to a higher level in VIGS *NaPLP* plants at 3 dpi, which indicates that biosynthesis of JA and ethylene is enhanced in *NaPLP*-silenced plants. Lipid metabolism is known to play an important role in plant defense response, and the *NaPLP* protein shares 65% sequence similarity to the potato tuber patatin with a strictly conserved serine hydrolase/lipase motif GXSXG (Fig. 1). This sequence similarity suggests that *NaPLP* may interfere with phospholipid-based signal transduction through nonspecific hydrolysis of phospholipids, which somehow directly or indirectly affects JA and ethylene biosynthesis, thereby reducing plant resistance to *A. alternata*.

Fig. 6. Synergistic induction of *NaPLP* by MeJA and ethephon is dependent on endogenous JA and ethylene signaling pathways. Mean (+s.e.) *NaPLP* transcripts were measured by real-time PCR in five biological replicates of 0 leaves in WT, irACO (JA-deficient), irCOI1 (JA-insensitive), irAOC (ethylene deficient), and Ov-etrl (ethylene-insensitive) plants co-treated with MeJA and ethephon at 24 and 72 dpi. Asterisks indicate the level of significant difference between each genotypes co-treated with MeJA and ethephon (Student’s t-test: *, p < 0.05; **, p < 0.01; ***, p < 0.005).
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