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Plant Diversity

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

Junbin Cheng [a, b], Na Song [a, b], Jinsong Wu [a, *]

[a] *Yunnan Key Laboratory for Wild Plant Resources, Kunming Institute of Botany, Chinese Academy of Sciences, Lanhei Road 132, 650201, Kunming, China*
[b] *University of Chinese Academy of Sciences, Beijing 10049, China*

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ABSTRACT

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 ethephon. Co-treatment with MeJA and ethephon 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.

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 hemi-biotrophic pathogens (Glazebrook, 2005). In response to *Alternaria alternata*, a notorious necrotrophic fungal pathogen causing blown 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.

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 (irCOI1) plants have been previously generated (von Dahl et al., 2007; Kallenbach et al., 2012;

The real time PCR primers for *NaACS1*, *NaACO1*, *NaAOS*, *NaLOX3* and *NaF6′H1* are listed below.

primers	Primer sequence 5′-3′	purpose	citation	Accession number
LOX3_F	TCGATCCTCACCACCCTCAT	*NaLOX3*	Sun et al. (2017)	AY254349
LOX3_R	CGGGATCATTCTCATCGGGG	*NaLOX3*	Sun et al. (2017)	AY254349
AOS_F	CGTACCCGATCGGTTCGTAG	*NaAOS*	Sun et al. (2017)	XM_019399851.1
AOS_R	ACCAGCACCACGAAATCCTT	*NaAOS*	Sun et al. (2017)	XM_019399851.1
JS142_F	CGAGTTATGGCTAATGGCAGC	*NaF6′H1*	Sun et al. (2014a,b)	KF771989
JS143_R	AGCACTTCAGCCAAAGGACC	*NaF6′H1*	Sun et al. (2014a,b)	KF771989
Sh33_Fr	GGATTCGGAGGTTTGTGGGT	*NaACS1*	Sun et al. (2017)	AY426752
Sh34_Rr	GAACGAGTGGTGAGTGAGGG	*NaACS1*	Sun et al. (2017)	AY426752
Sh39_Fr	CTATTGAATCTGATGTCAAGCTG	*NaACO1*	von Dahl et al. (2007)	AY426756
Sh40_Rr	TATGTAGTAGGGACACACGCTT	*NaACO1*	von Dahl et al. (2007)	AY426756

Paschold et al., 2007) and were provided by Prof. Ian T. Baldwin (Max-Planck Institute for Chemical Ecology, Jena, Germany). Seed germination and plant growth were conducted as described in Krügel et al. (2002). *A. alternata* were grown and used for inoculation as described in Sun et al. (2014a).

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: 5′-CAT-AAGCTTCCCTTCTCTGCTGCCAAAG-3′ and CJ08: 5′-CATGGATCCG-CAGCAACAACACCACCATC-3′), and subsequently cloned into pTV00. To generate *NaPLP*-silenced plants (VIGS NaPLP), *Agrobacterium tumefaciens* (GV3101) cells carrying the above construct were combined with cells having pBINTRA, and were inoculated into leaves of *N. attenuata*. Green tissue of *Phytoene desaturase* (PDS)-silenced plants shows visible bleaching two to three weeks after transformation (Saedler and Baldwin, 2004; Wu et al., 2008), thus they were used as positive controls. When *PDS*-silenced plants began to bleach, the young rosette leaves of VIGS NaPLP and empty vector-transformed plants (EV plants) were collected for experiments. All VIGS experiments were repeated twice.

2.3. Methyl jasmonate (MeJA) and ethephon treatments

Methyl jasmonate (MeJA) and ethephon treatments were conducted as described in Xu et al. (2018). Briefly, 1 mM MeJA (www.sigmaaldrich.com) and 5 mM ethephon (www.sangon.com) were prepared using distilled water, and sprayed with a fine mist individually or simultaneously on the source-sink transition leaves (young 0 leaves) of 35-day-old, rosette-staged *N. attenuata* plants

until run off. The treated plants were immediately covered with a plastic bag, and harvested after 1, 3, 6, 12, 24, and 72 h. For control treatments, plants were sprayed with distilled water.

2.4. Real-time PCR assay

Total RNA of 4–5 replicate biological samples was extracted from a 1.5×1.5 cm^2 area of leaf lamina with the inoculation site at the center. cDNA was synthesized from 500 ng total RNA with reverse transcriptase (Thermo Scientific). Real-time PCR was performed on a CFX Connect qPCR System (Bio-Rad) by using the iTaq Universal SYBR Green Supermix (Bio-Rad) and gene-specific primers (CJ05: 5′- TGCTGGGACATACACAGCAG 3′; CJ06: 5′- CCAA-GAGCGCGATAGATGGT 3′) as described by Wu et al. (2013). Transcript abundance of *NaActin 2* was not altered either in leaves inoculated with *A. alternata* at 1 and 3 dpi or in leaves treated with MeJA and ethephon, and thus was used as an internal standard (Xu et al., 2018).
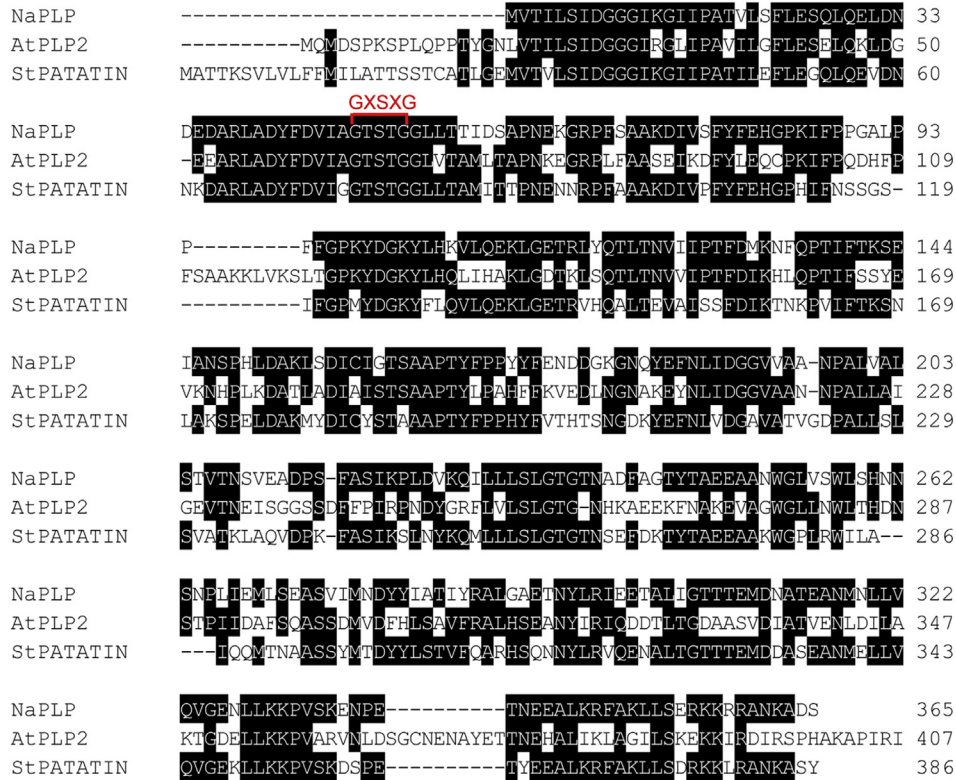
3. Results

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 *Patatin-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 GXSXG (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 NaPLP and EV plants to investigate whether *NaPLP*-silencing affects *N. attenuata* resistance to *A. alternata*. The lesion diameter in

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NaPLP       --------------------------MVTILSIDGGGIKGIIPATVLSFLESQLQELDN    33
AtPLP2      ----------MQMDSPKSPLQPPTYGNIVTILSIDGGGIRGLIPAVILGFLESELQKLDG    50
StPATATIN   MATTKSVLVLFFMILATTSSTCATLGEMVTLSIDGGGIKGIIPATIIEFLEGQLQEVDN    60
                                GXSXG
NaPLP       DEDARLADYFDVIAGTSTGGLLTTIDSAPNEKGRPFSAAKDIVSFYFEHGPKIFBPGALP    93
AtPLP2      -BEARLADYFDVIAGTSTGGLVTAMLTAPNKEGRPLFAASEIKDFYLEQQPKIFEQDHFP   109
StPATATIN   NKDARLADYFDVIGGTSTGGLLTAMITTPNENNRPFAAAKDIVPFYFEHGPHIENSSGS-   119

NaPLP       P---------FFGPKYDGKYLHKVLQEKLGETRIYQTLTNVIIPTFDMKNFQPTIFTKSE   144
AtPLP2      FSAAKKLVKSLTGPKYDGKYLHQLIHAKLGDTKLSQTLTNVVIPTFDIKHLQPTIESSYE   169
StPATATIN   ---------IFGPMYDGKYFLQVLQEKLGETRVHQALTEVAISSFDIKTNKPVIFTKSN   169

NaPLP       IANSPHLDAKLSDICIGTSAAPTYFPEYYFENDBGKGNQYEFNLIDGGVVAA-NPAIVAL   203
AtPLP2      VKNHELKDATLADIAISTSAAPTYLPAHFFKVEDINGNAKEYNLIDGGVAAN-NPALLAI   228
StPATATIN   LAKSPELDAKMYDICYSTAAAPTYFPPHYFVTHTSNGDKYEFNIVDGAVATVGDPALLSL   229

NaPLP       STVTNSVEADPS-FASIKPLDVKQILLLSLGTGTNADFAGTYTAEEAANWGLVSWISHNN   262
AtPLP2      GEVTNEISGGSSDFFPIRFNDYGRFLVLSLGTG-NHKAEEKFNAKEVAGWGLLNWITHDN   287
StPATATIN   SVATKLAQVDPK-FASIKSLNYKQMLLLSLGTGTNSEFDKTYTAEEAAKWGPLRWILA--   286

NaPLP       SNPLIEMLSEASVIMNDYYIATIYRALGAETNYLRIEETALIGTTTEMDNATEANMNLLV   322
AtPLP2      STPIIDAFSQASSDMVDFHLSAVFRALHSEANYIRIQDDTLTGDAASVDIATVENLDILA   347
StPATATIN   ---IQQMTNAASSYMTDYYLSTVFQARHSQNNYLRVQENALTGTTTEMDDASEANMELLV   343

NaPLP       QVGENLLKKPVSKENPE---------TNEEALKRFAKLLSERKKRRANKADS          365
AtPLP2      KTGDELLKKPVARVNLDSGCNENAYETTNEHALIKLAGILSKEKKIRDIRSPHAKAPIRI   407
StPATATIN   QVGEKLLKKPVSKDSPE---------TYEEALKRFAKLLSDRKKLRANKASY          386
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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*, CAA25592.1). Consensus sequences are shaded with black and the serine hydrolase GXSXG motif is indicated in red font (bracket).

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
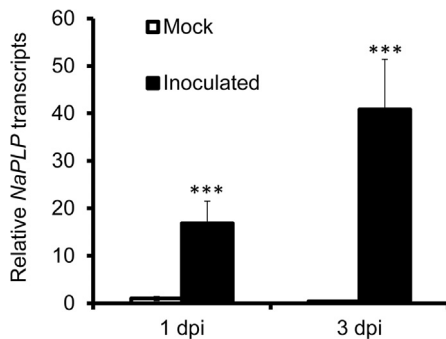
Fig. 2. Elicitation of *NaPLP* by *A. alternata* in WT plants. *NaPLP* transcripts were measured by real-time PCR in the source-sink transition leaves (0 leaves) of WT plants treated with mock or with *A. alternata* at 1 and 3 days post inoculation (dpi). All transcriptional levels were normalized with a housekeeping gene *NaActin II*. Values are means $\pm$ 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: ***, $p < 0.005$).
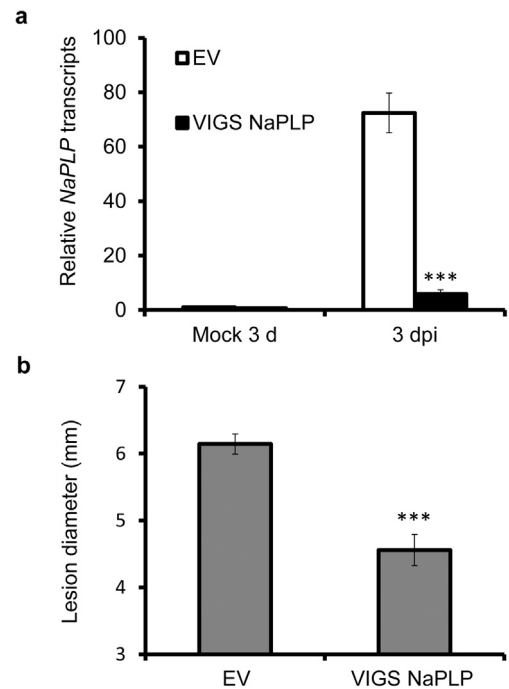
Fig. 3. Silencing *NaPLP* enhances plant resistance to *A. alternata*. a) Mean ($\pm$SE) *NaPLP* transcript levels were measured by real-time PCR in five biological replicates of young leaves of EV and VIGS NaPLP plants at 3 dpi. b) Mean ($\pm$SE) diameter of necrotic lesions of fifteen replicates of 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: *, $p < 0.05$; ***, $p < 0.005$).
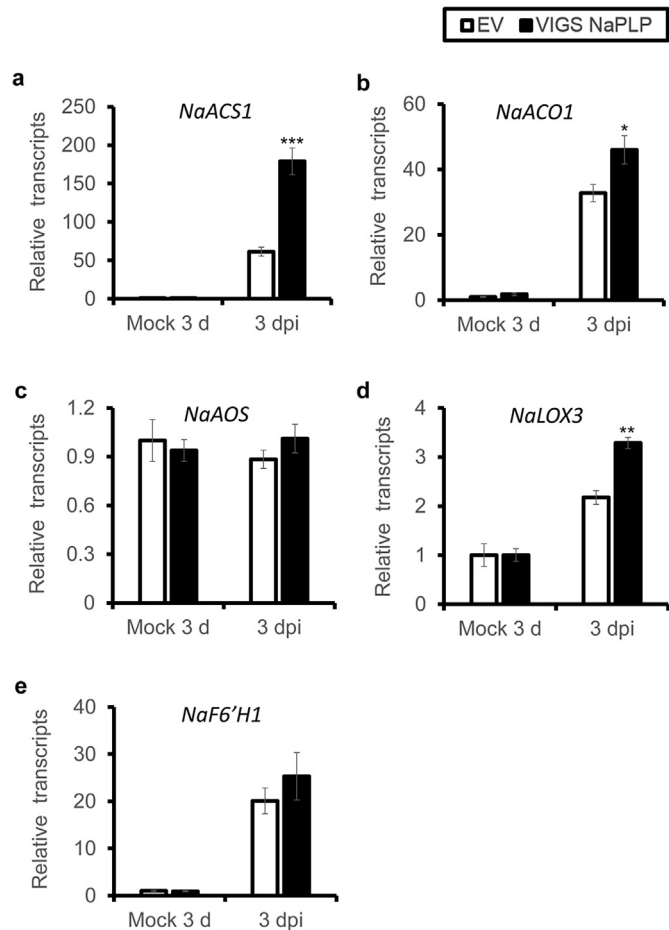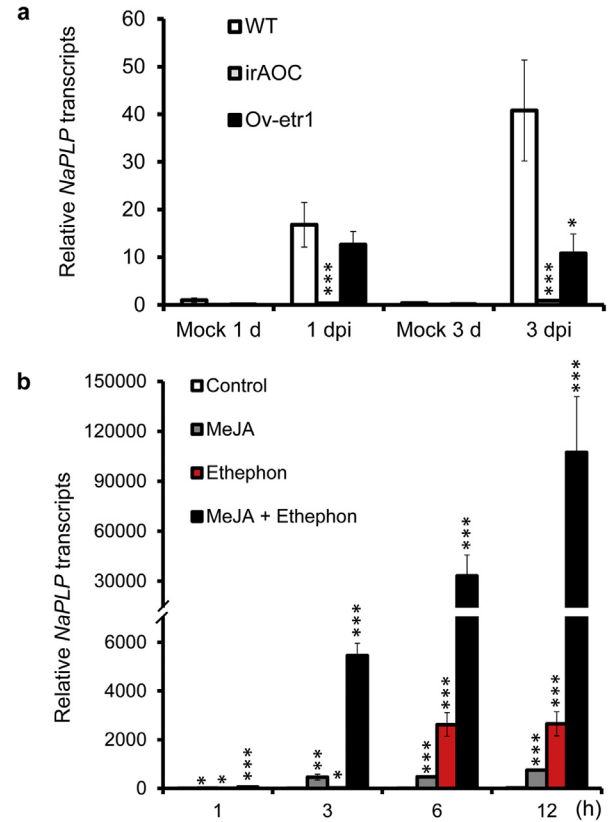
Fig. 4. Effects of Silencing *NaPLP* on gene expression of JA and ethylene biosynthetic genes. Mean (±SE) *NaACS1* (a), *NaACO1* (b), *NaAOS* (c), *NaLOX3* (d), *NaF6'H1* (e) transcript levels were measured by real-time PCR in five biological replicates of young leaves of EV and VIGS NaPLP plants at 3 dpi. Asterisks indicate the level of significant difference between control and treated leaves (Student's *t*-test: *, p < 0.05; **, p < 0.01; ***, p < 0.005).


Fig. 5. Elicitation of *NaPLP* transcripts by *A. alternata* in JA-deficient and ethylene-insensitive plants; and by methyl jasmonate and ethephon in WT. *a*) *NaPLP* transcripts were measured by real-time PCR in the source-sink transition leaves (0 leaves) of WT, irAOC (JA-deficient) and Ov-etr1 (ethylene-insensitive) plants treated with mock or with *A. alternata* at 1 and 3 days post inoculation (dpi). b) Mean (±SE) *NaPLP* transcripts were measured by real-time PCR in five replicates of 0 leaves treated with water (as control), MeJA, and Ethephon individually and simultaneously at 1, 3, 6, and 12 h. Asterisks indicate the level of significant difference between control and treated leaves (Student's *t*-test: *, p < 0.05; **, p < 0.01; ***, p < 0.005).

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.
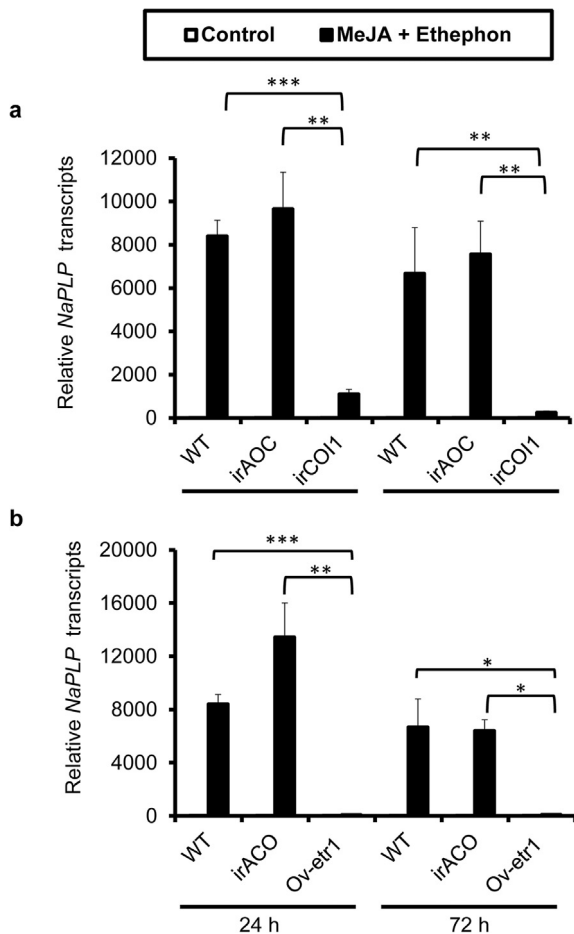
Fig. 6. Synergistic induction of *NaPLP* by MeJA and ethephon is dependent on endogenous JA and ethylene signaling pathways. Mean (±SE) *NaPLP* transcripts were measured by real-time PCR in five biological replicates of 0 leaves in WT, irAOC (JA-deficient), irCOI1 (JA-insensitive), irACO (ethylene deficient), and Ov-etr1 (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).

4. Discussion

Although patatin was initially identified as a major storage proteins, 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*.

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 *NaF6′H1*, 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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