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Plant Diversity

NaPDR1 and NaPDR1-like are essential for the resistance of Nicotiana attenuata against fungal pathogen Alternaria alternata

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ABSTRACT

Pleiotropic drug resistance (PDR) transporters are widely distributed membrane proteins catalyzing the export or import of a diverse array of molecules, and are involved in many plant responses to biotic and abiotic stresses. However, it is unclear whether PDRs are involved in Nicotiana attenuata resistance to the necrotic fungal pathogen Alternaria alternata. In this study, transcriptional levels of both NaPDR1 and NaPDR1-like were highly induced in N. attenuata leaves after A. alternata inoculation. Interestingly, silencing NaPDR1 or NaPDR1-like individually had little effect on N. attenuata resistance to A. alternata; however, when both genes were co-silenced plants became highly susceptible to the fungus, which was associated with elevated JA and ethylene responses. Neither NaPDR1 nor NaPDR1-like was significantly elicited by exogenous treatment with methyl jasmonate (MeJA), whereas both were highly induced by ethylene. The elicitation levels of both genes by A. alternata were significantly reduced in plants with impaired JA or ethylene signaling pathways. Thus, we conclude that both NaPDR1 and NaPDR1-like function redundantly to confer resistance against A. alternata in N. attenuata, and the elicitation of the transcripts of both genes by the fungus is partially dependent on ethylene and jasmonate signaling.

Introduction

The ATP-binding cassette (ABC) transporters belong to a large protein superfamily present in all living organisms, and are involved in the transportation of a wide range of molecules across membranes when energized by ATP hydrolysis (Lefevre et al., 2015; Nuruzzaman et al., 2014). The ABC transporter family has been divided according to structural features into full-size and half-size transporters. One of the well-known full-size subfamilies is the pleiotropic drug resistance (PDR) transporters.

In plants, PDR transporters constitute one of the biggest sub-families of ABC transporters, and have recently been shown to perform critical physiological functions in plant response to biotic stresses (van den Brule et al., 2002; Lefevre et al., 2015; Nuruzzaman et al., 2014). PDR2 in Petunia hybrid and PDR5 in Nicotiana tabacum are involved in herbivore defense (Sasse et al., 2016; Bienert et al., 2012). In Nicotiana plumbaginifolia, NpPDR1 is induced by biotic stresses; and silencing NpPDR1 leads to plants highly susceptible to a group of fungal and oomycete pathogens which include Botrytis cinerea and Phytophthora nicotianae race 0 (Bultreys et al., 2009; Stukkens et al., 2005). PENETRATION (PEN) 3 (also called PDR8 or ABCG36) is required for Arabidopsis non-host resistance (Stein et al., 2006). Evidence also suggests that PDR transporters (e.g. AtPDR12 and NtPDR1) function in resistance against bacterial wilt disease in tobacco and Arabidopsis plants (Seo et al., 2012). However, it is not clear if PDR transporters play a role in plant defense in Nicotiana attenuata—Alternaria alternata (tobacco pathotype) interaction.

N. attenuata is an annual wild diploid tobacco native to western North America, and has been extensively used as a host for studying

herbivore-elicited responses (Wu and Baldwin, 2010). Similar to cultivated tobacco, *N. attenuata* is attacked by *A. alternata*, a notorious necrotrophic fungus that causes brown spot disease in tobacco species in the field (Schuck et al., 2014). In response to this fungal infection, *N. attenuata* plants activate abscisic acid (ABA)-mediated stomatal defense (Sun et al., 2014a), as well as the jasmonate (JA) and ethylene signaling pathways (Sun et al., 2014b, 2017; Li and Wu, 2016).

In this study, two PDRs, which were up-regulated after *A. alternata* inoculation, were selected to test their roles in plant resistance via a virus-induced gene silencing technique, and the regulation of the transcriptional levels of these two genes by JA and ethylene was also investigated.

Materials and methods

Plant and fungal material

Seeds of the 33rd generation of an inbred line of *N. attenuata* (Solanaceae) were defined as the wild type (WT) genotype in experiments. Stably transformed lines of irAOC (Kallenbach et al., 2012), irACO (von Dahl et al., 2007) and Ov-etr1 (von Dahl et al., 2007) were provided by Professor Ian T. Baldwin (Max-Planck Institute for Chemical Ecology, Jena, Germany). Seed germination and plant growth were conducted as described by Krugel et al. (2002).

A. alternata was grown and inoculated as described by Sun et al. (2014b).

Methyl jasmonate (MeJA) and Ethephon treatments

MeJA treatment is the most commonly used means of eliciting JA-regulated responses in plants; when applied, the chemical is quickly de-methylated into jasmonic acid by plant MeJA-esterase (Wu et al., 2008). Ethephon is one of the most widely used synthetic plant growth regulators, and is converted into ethylene, thereby eliciting ethylene-induced responses in plants. 1 mM MeJA (www.sigmaaldrich.com) and 5 mM ethephon (www.sangon.com) were prepared by distilled water, and sprayed directly on the source−sink transition leaves (0 leaves) of 35-day-old rosette staged *N. attenuata* plants until run off with a fine mist of either compound at the indicated concentrations. The treated plants were immediately covered with a plastic bag, and harvested after 1, 3, 6, 12 h. Distilled water was sprayed and used as a control treatment.

Generation of VIGS plants

Specific 137-bp PCR fragment used for silencing *NaPDR1* (XM_019378156) was amplified by the primer pair XZ31 and XZ32 (all primers used in this study were included in Supplementary table). The 135-bp specific PCR fragment used for silencing *NaPDR1-like* (XM_019396612) was cloned by primer pair XZ35 and XZ36. In addition, a 188-bp PCR fragment used for silencing *NaPDR1* and *NaPDR1-like* together was amplified by primers ABC01 and ABC02. All PCR products were cloned into pTV00 through *Bam*H I and *Hind* III respectively. To generate *NaPDR1-* (VIGS PDR1 plant) and *NaPDR1-like*-silenced plants (VIGS PDR1-like plant), as well as *NaPDR1* and *NaPDR1-like*-co-silenced plants (VIGS PDR1 and 1-like plant), *Agrobacterium tumefaciens* GV3101 cells carrying the above individual construct were combined with those cells having pBINTRA, and were inoculated into 25-day-old *N. attenuata* leaves through 1 mL syringe without a needle.

To monitor the progress of VIGS, we silenced *phytoene desaturase* (*PDS*), which causes plants to show visible bleaching of green tissues two to three weeks after transformation (Saedler and

Baldwin, 2004; Wu et al., 2008; Sun et al., 2017). When the leaves of *PDS*-silenced plants began to bleach, the source−sink transition leaves of EV and VIGS plants were used for experiments. Around 20 plants were inoculated with each construct, and all VIGS experiments were repeated twice.

Real-time PCR assay

Total RNA was extracted from leaf lamina in an approximately 1.5 by 1.5 cm^2 area which encompassed the inoculation site. The preparation of total RNA and cDNA was performed as described by Wu et al. (2013). Real-time PCR was conducted on a CFX Connect qPCR System (Bio-Rad) with iTaq Universal SYBR Green Supermix (Bio-Rad) and gene-specific primers according to the manufacturer's instructions. For each analysis, a linear standard curve obtained from threshold cycle number versus log DNA quantity was constructed by using a dilution series of a specific cDNA sample, and the levels of the transcript in all unknown samples were calculated according to the standard curve. Finally, relative transcript levels of target genes were obtained by dividing the extrapolated transcript levels of the target genes by the levels of a housekeeping gene *Actin II* as an internal standard from the same sample. The expression level of housekeeping gene *Actin II* was not altered in leaves either inoculated with *A. alternata* at 1 and 3 dpi, or treated with MeJA (or Ethephone) at 1 and 3 h (Supplementary data 1).

Statistical analyses

All samples represent biological replicates. Sample sizes are indicated in the figure legends. Unpaired two-tailed Student's *t*-tests and ANOVA were used to compare sets of data obtained from different treatments as indicated in figure legends. All experiments were repeated twice.

Results

Elicitation of NaPDR1 and NaPDR1-like transcripts by A. alternata

Transcriptome analysis revealed that after source−sink transition leaves (0 leaves) were inoculated with *A. alternata* for one day, two pleiotropic drug resistance transporter genes (*NaPDR1* and *NaPCR1-like*) were up-regulated. To confirm these data, we quantified *NaPDR1* (Gene bank accession number: XM_019378156) and *NaPDR1-like* gene (Gene bank accession number: XM_019396612) expression levels using real-time PCR analysis. Both *NaPDR1* and *NaPDR1-like* expression was strongly elicited in 0 leaves at one day post inoculation (1 dpi), and reached its highest levels at 3 dpi (Fig. 1).

Silencing NaPDR1 or NaPDR1-like individually had no effect on N. attenuata resistance to A. alternata

To study the role of *NaPDR1* or *NaPDR1-like* in *N. attenuata* resistance to *A. alternata*, both genes were silenced individually by virus-induced gene silencing (VIGS). Three days after inoculation with *A. alternata*, plants transformed with *NaPDR1* VIGS constructs (VIGS PDR1 plants) had only 26% of the *NaPDR1* transcripts that plants transformed with empty vector (EV plant) or *NaPDR1-like* VIGS constructs (VIGS PDR1-like plant) had, suggesting *NaPDR1* was successfully silenced in VIGS NaPDR1 plants without affecting the transcriptional level of *NaPDR1-like* (Fig. 2a). VIGS PDR1 plants inoculated with *A. alternata* developed necrotic lesions in 0 leaves of about the same size as EV plants (Fig. 2b). This result indicates that silencing *NaPDR1* did not affect *N. attenuata* resistance to *A. alternata*. The transcriptional level of *A. alternata*-elicited
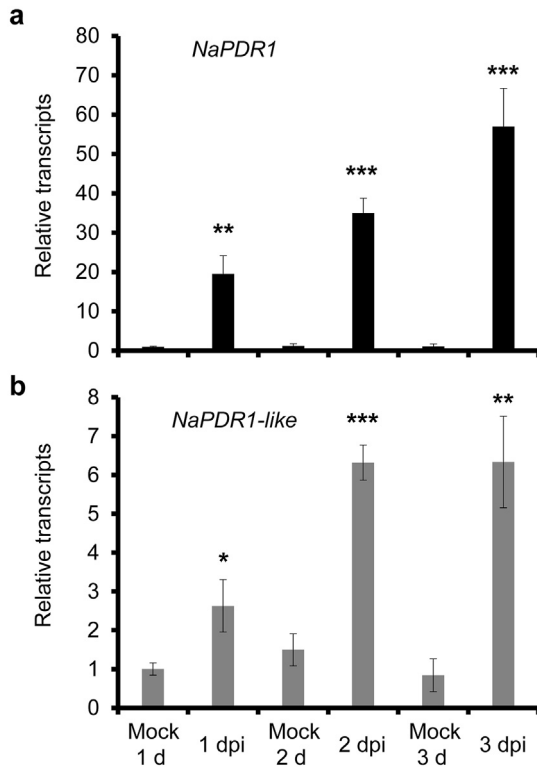
Fig. 1. Elicitation of *NaPDR1* and *NaPDR1-like* after *A. alternata* inoculation. *NaPDR1* (a) or *NaPDR1-like* (b) transcripts were measured by real-time PCR in 0 leaves treated with mock or with *A. alternata* at 1, 2, and 3 days post inoculation (dpi). All transcriptional levels were normalized with a housekeeping gene *Actin II*. Values are means ± SE for fives replicates. Asterisks indicate the level of significant difference between mock and infected leaves (Student's *t*-test: *, P < 0.05; **, P < 0.01; ***, P < 0.005).

Fig. 2. Silencing *NaPDR1* or *NaPDR1-like* individually had no effect on *N. attenuata* resistance to *A. alternata*. a) Mean (±SE) *NaPDR1* and *NaPDR1-like* transcripts were measured by real-time PCR in ten replicates of 0 leaves of EV, VIGS PDR1 and VIGS PDR1-like plants at 3 dpi. b) Mean (±SE) diameter of necrotic lesions of fifteen replicates of 0 leaves of EV, VIGS PDR1 and VIGS PDR1-like plants at 6 dpi. Asterisks indicate the level of significant difference between *A. alternata*-elicited EV and VIGS plants with the same treatments (Student's *t*-test: **, P < 0.01; ***P < 0.005).

NaPDR1-like in VIGS PDR1-like plants was silenced by 79% compared with EV or VIGS NaPDR1 plants (Fig. 2a). VIGS PDR1-like plants developed slightly smaller necrotic lesions in 0 leaves compared to EV or VIGS NaPDR1 plants, although the differences were not significant (Fig. 2b). All these data suggest that silencing *NaPDR1* or *NaPDR1-like* individually does not affect *N. attenuata* resistance to *A. alternata*.

Enhanced susceptibility of N. attenuata plants to A. alternata by co-silencing NaPDR1 and NaPDR1-like

NaPDR1 and NaPDR1-like proteins share high sequence similarity (Supplementary data 1). To investigate whether they function redundantly in *N. attenuata* resistance to *A. alternata*, we co-silenced *NaPDR1* and *NaPDR1-like*. When both *NaPDR1* and *NaPDR1-like* genes were successfully silenced (Fig. 3a and b), necrotic lesion diameter at 6 dpi doubled (8.25 cm) in 0 leaves compared to those with the same leaf position in EV plants (4 cm) (Fig. 3c and d). This indicates that plants co-silenced for *NaPDR1* and *NaPDR1-like* are more susceptible to *A. alternata*.

JA and ethylene biosynthetic genes were elicited by A. alternata in NaPDR-silenced plants

We have previously shown that both JA and ethylene signaling pathways are activated in *N. attenuata* plants after *A. alternata* inoculation. Furthermore, JA-deficient and ethylene-insensitive plants develop larger lesions than WT plants after inoculation with *A. alternata* (Sun et al., 2014b, 2017; Li and Wu, 2016). These findings
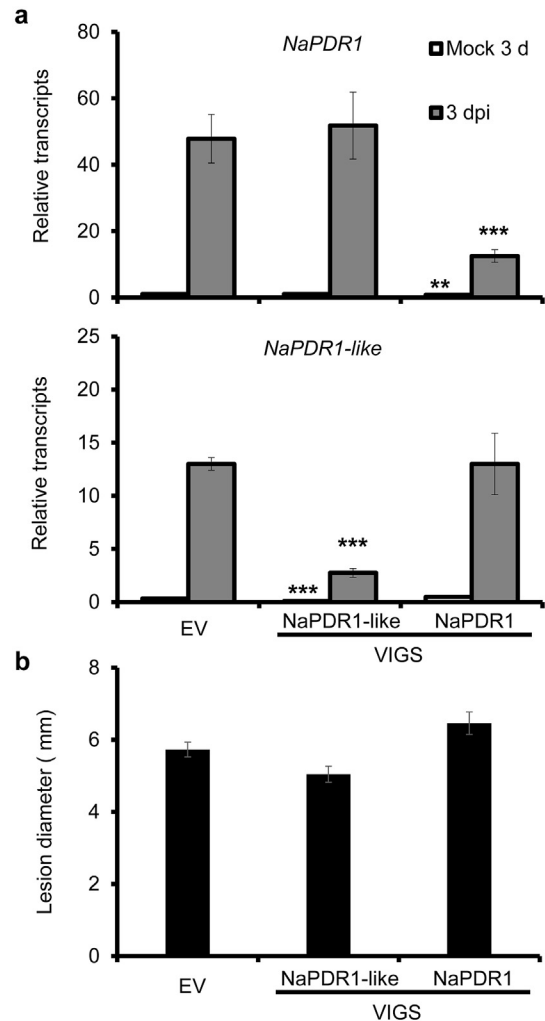
indicate that JA and ethylene signaling pathways play important roles in host defense response. To investigate whether the high susceptibility of *NaPDR1* and *NaPDR1-like* co-silenced plants to *A. alternata* was due to inhibition of the JA and ethylene signaling pathways, we examined the transcriptional levels of three JA and ethylene biosynthetic genes (*NaLOX3*, *NaACS1*, and *NaACO1*) (Halitschke and Baldwin, 2003; Sun et al., 2017) at 3 dpi, when PDRs were highly expressed. As expected, all three genes were up-regulated in WT plants at 3 dpi (Fig. 4). However, their transcripts were elicited to a higher level in VIGS PDR1, VIGS PDR1-like, and VIGS PDR1 and 1-like plants (Fig. 4). For *NaACS1*, even higher levels were elicited in leaves of VIGS PDR1 and 1-like plants (Fig. 4). These results suggest that the JA and ethylene signaling pathways were activated at higher levels in PDR-silenced plants.

Involvement of jasmonate (JA) and ethylene signaling pathways in the elicitation of NaPDR1 and NaPDR1-like by A. alternata

Neither *NaPDR1* nor *NaPDR1-like* was significantly induced by exogenous methyl jasmonate (MeJA) (Fig. 5). Both genes were
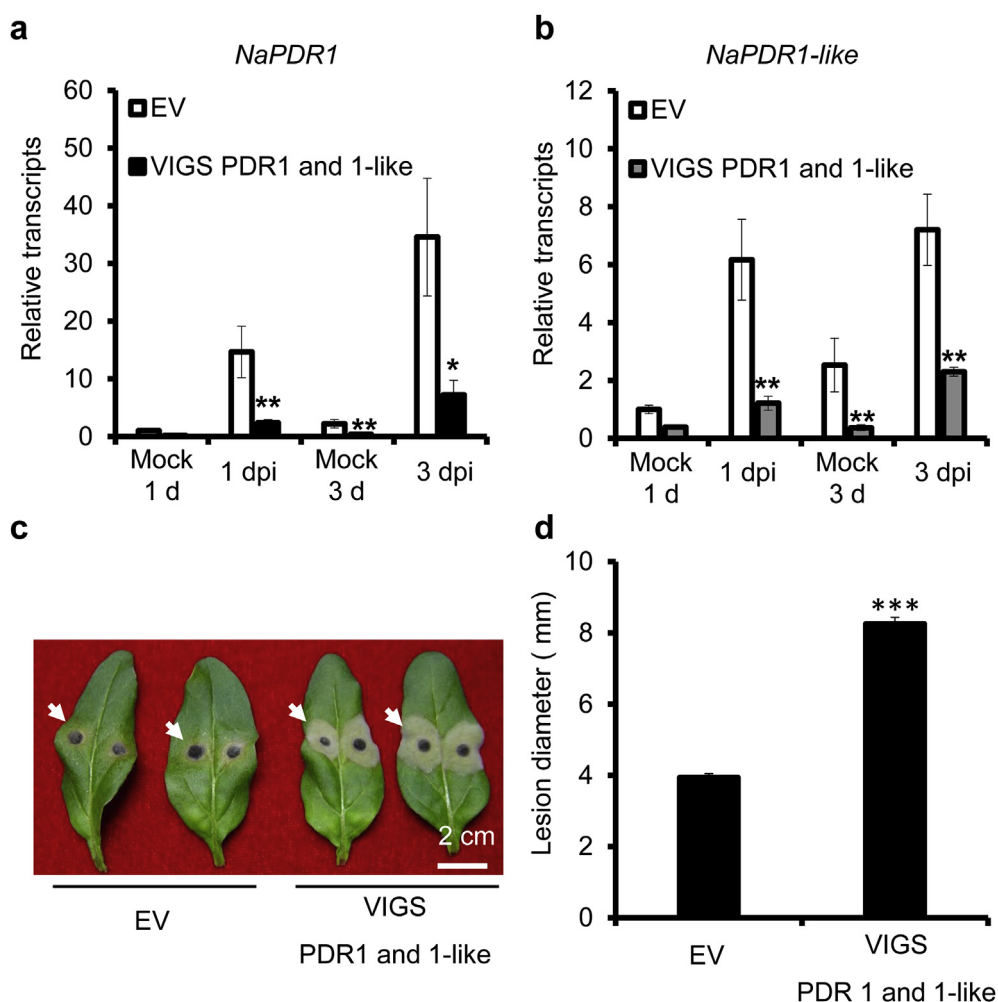
Fig. 3. Co-silencing *NaPDR1-like* and *NaPDR1* lead to plants highly susceptible to *A. alternata*. a) Mean (±SE) *NaPDR1* transcripts were measured by real-time PCR in six replicates of 0 leaves of EV and VIGS PDR1 and 1-like plants at 1 dpi and 3 dpi. b) Mean (±SE) *NaPDR1-like* transcripts were measured by Real-time PCR in six replicates of EV and VIGS PDR1 and 1-like plants at 1 dpi and 3 dpi. c) Photographs were taken of two 0 leaves of EV plants and two 0 leaves of VIGS PDR1 and 1-like plants at 6 dpi. White arrows indicate lesions. d) Mean (±SE) diameter of necrotic lesions of fifteen replicates of 0 leaves of EV, VIGS PDR1 and 1-like plants at 6 dpi. Asterisks indicate the level of significant differences between EV and VIGS PDR1 and 1-like plants (Student's *t*-test: *, P < 0.05; **, P < 0.01; ***, P < 0.005).

strongly elicited after plants were treated with ethephon, a synthetic compound which decomposes into ethylene upon metabolism by plants (Fig. 5). These results indicate that ethylene signaling might play a role in the regulation of *NaPDR1* and *NaPDR1-like* transcripts. We thus tested whether *A. alternata*-elicited transcripts of both genes decreased in plants in which allene oxide cyclase was silenced (JA-deficient irAOC plants), and plants that over-expressed an *Arabidopsis* ethylene receptor mutant *etr1-1* gene (Ov-etr1 plants are insensitive to exogenous ethylene) (Sun et al., 2014b, 2017; Kallenbach et al., 2012; von Dahl et al., 2007). As expected, both *NaPDR1* and *NaPDR1-like* transcript levels decreased significantly in irAOC, irACO and Ov-etr1 plants at 1 dpi but not 3 dpi (Fig. 6), indicating that both JA and ethylene signaling are only partially involved in the elicitation of *NaPDR1* and *NaPDR1-like* transcripts by *A. alternata*.

Discussion

An increasing number of reports have shown that PDRs are involved in plant resistance against pathogens (Bultreys et al., 2009; Stukkens et al., 2005; Stein et al., 2006; Seo et al., 2012; Shibata et al., 2016). Our results strongly support the idea that

both *NaPDR1* and *NaPDR1-like* play important roles in *N. attenuata* resistance to *A. alternata*. We found that *NaPDR1* and *NaPDR1-like* were both strongly elicited by *A. alternata* inoculation. In addition, co-silencing *NaPDR1* and *NaPDR1-like* increased the susceptibility of *N. attenuata* plants to *A. alternata*. However, silencing *NaPDR1* and *NaPDR1-like* individually did not affect *A. alternata*-induced lesion size after inoculation, indicating that both genes function redundantly during plant resistance to the fungus.

The JA and ethylene pathways are two of the most important phytohormonal signaling pathways involved in plant resistance to necrotrophic pathogens (Glazebrook, 2005; Mengiste, 2012; Campos et al., 2014). Indeed, *N. attenuata* plants strongly impaired in JA/ethylene biosynthesis or perception have been shown to be highly susceptible to the necrotrophic fungal pathogen *A. alternata* (Sun et al., 2014b, 2017). In this study, fungal inoculation of *NaPDR1* and *NaPDR1-like* co-silenced plants increased transcriptional levels of *NaLOX3*, *NaACS1* and *NaACO1*, three key enzyme genes strongly responsive to exogenous JA or ethylene respectively. This indicates that both JA and ethylene signalling were strongly elicited in VIGS PDR1 and VIGS PDR1-like plants. Thus, the increased susceptibility of NaPDR1 and NaPDR1-like co-silenced plants to *A. alternata* was not because the JA or ethylene signaling pathways were blocked.

Many phytohormones and pathogens are involved in the regulation of PDR genes expression. This is the case for *NtPDR5*, which is induced by MeJA and *P. nicotianae* (Bienert et al., 2012). We found that *NaPDR1* and *NaPDR1-like* transcripts were strongly induced after *A. alternata* inoculation. When plants were treated with ethephon, high levels of *NaPDR1* and *NaPDR1-like* transcripts were elicited, whereas they were not significantly induced after MeJA treatments. These data suggest that the elicitation of *NaPDR1* and *NaPDR1-like* transcripts by *A. alternata* may be regulated by the ethylene signaling pathway. The fact that the levels of *A. alternata*-elicited transcripts of *NaPDR1* and *NaPDR1-like* were decreased in JA-deficient (irAOC) or ethylene-insensitive (Ov-etr1) plants at 1 dpi but not at 3 dpi indicates that the fungus-induced both PDR transcripts were only partially depended on the endogenous JA and ethylene signaling pathways.

It is not clear why *NaPDR1* and *NaPDR1-like* co-silenced plants showed increased susceptibility to *A. alternata*. One possibility is that these plants may be unable to transport important 'chemical weapons' required for defense against the fungus, similar to how *Nicotiana benthamiana* plants inoculated with *Phytophthora infestans* are unable to transport capsidiol (Shibata et al., 2016). Alternatively, when inoculated with *A. alternata* plants co-silenced with *NaPDR1* and *NaPDR1-like* may simply get more stressed because
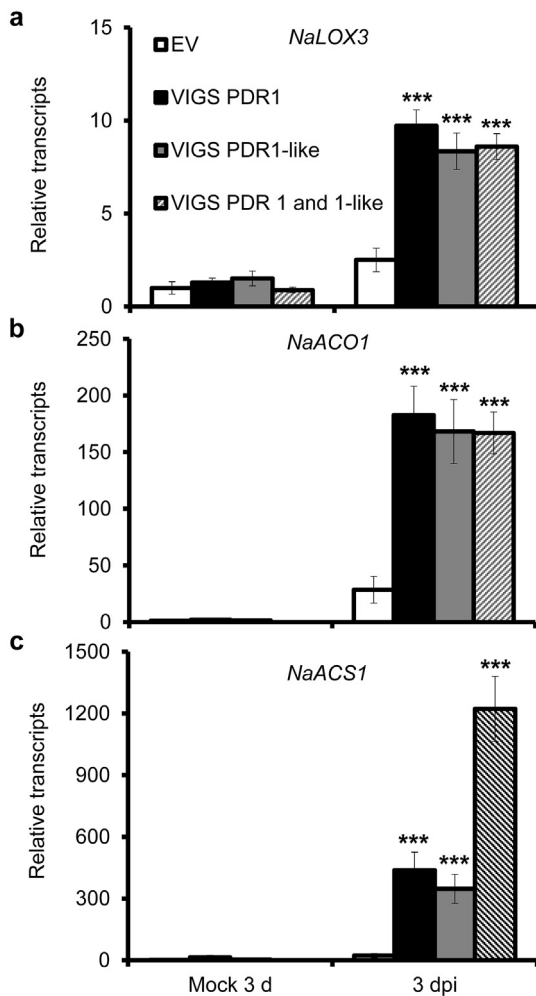

Fig. 5. Elicitation of *NaPDR1-like* and *NaPDR1* transcripts by methyl jasmonate and ethephon. Mean (±SE) *NaPDR1* (a) and *NaPDR1-like* (b) transcripts were measured by real-time PCR in five replicates of 0 leaves treated with water (as control), MeJA and Ethephon at 1, 3, 6, and 12 h. The different letters indicate the level of significant difference (Tukey's HSD test, P < 0.05).

Fig. 4. Elicitation of *NaLOX3*, *NaACS1*, *NaACO1* by *A. alternata* in PDR-silenced plants. Mean (±SE) *NaLOX3* (a) *NaACO1* (b) and *NaACS1* (c) transcripts were measured by real-time PCR in five replicates of 0 leaves at 3 dpi. Asterisks indicate the level of significant difference between EV and VIGS plants at 3 dpi (Student's *t*-test: ***, P < 0.005).
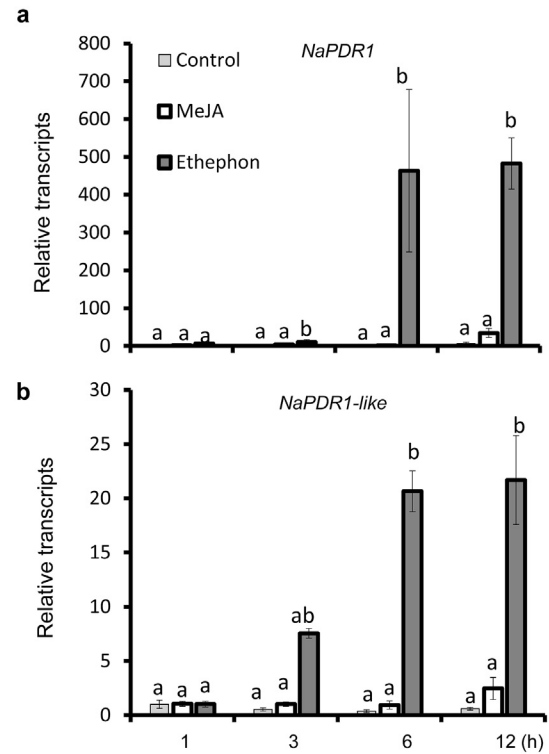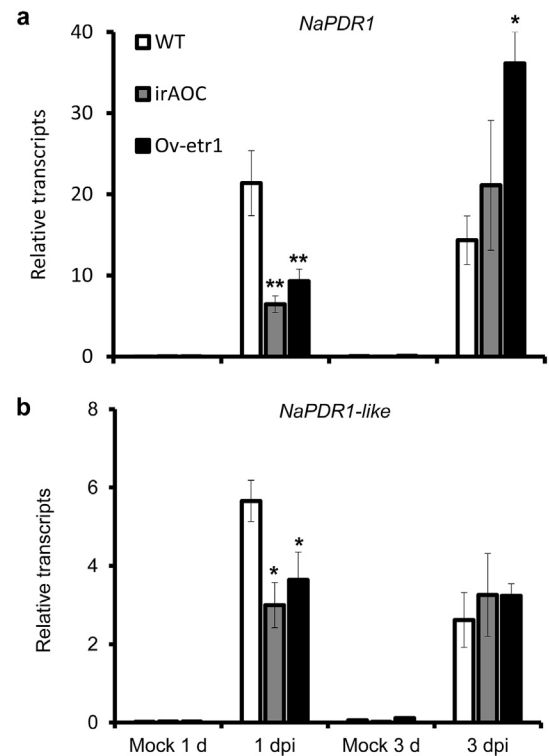

Fig. 6. Reduction of *A. alternata*-induced *NaPDR1-like* and *NaPDR1* transcripts at 1 dpi but not 3 dpi in plants impaired with JA or ethylene signaling pathway. Mean (±SE) *NaPDR1* (a) and *NaPDR1-like* (b) transcripts were measured by real-time PCR in five replicates of 0 leaves treated with mock or *A. alternata* at 1 and 3 dpi. Asterisks indicate the level of significant difference between WT and transgenic plants (Student's *t*-test: *, P < 0.05; **, P < 0.01).

they are unable to transport important metabolites which are currently unknown, and those stresses lead to plants more vulnerable to *A. alternata*.

Taken together, our results indicate that both *NaPDR1* and *NaPDR1-like* function redundantly, and play important roles in *N. attenuata* resistance to *A. alternata*. Both PDR transcripts were highly elicited after fungal inoculation, which appears to be partially dependent on JA and ethylene signaling pathways.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pld.2018.01.001.

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