Plant Pathology (2017) **66**, 277–284 Doi: 10.1111/ppa.12568

Ethylene signalling is essential for the resistance of *Nicotiana attenuata* against *Alternaria alternata* and phytoalexin scopoletin biosynthesis

H. Sun[ab†], N. Song[a†], L. Ma[a], J. Li[ab], L. Ma[a], J. Wu[a] and J. Wu[a*]

[a]*Key Laboratory of Economic Plants and Biotechnology, Yunnan Key Laboratory for Research and Development of Wild Plant Resources, Kunming Institute of Botany, Chinese Academy of Sciences, Lanhei Road 132, 650201 Kunming; and* [b]*University of Chinese Academy of Sciences, Beijing 100049, China*

The phytohormone ethylene plays an important role in plant defence responses to pathogen attack. When infected by the necrotrophic fungal pathogen *Alternaria alternata* (tobacco pathotype), which causes severe diseases in *Nicotiana* species, the wild tobacco plant *Nicotiana attenuata* accumulates a high amount of the jasmonate (JA)-dependent phytoalexin scopoletin to defend itself against this fungal pathogen. However, it is still not known whether ethylene signalling is also involved in scopoletin biosynthesis and the resistance of *N. attenuata*. After infection, ethylene biosynthetic genes were highly elicited. Furthermore, plants strongly impaired in ethylene biosynthesis or perception had dramatically decreased scopoletin levels, and these plants became more susceptible to the fungus, while *A. alternata*-elicited JA levels were increased, indicating that the decreased defence responses were not due to lower JA levels. Thus, it is concluded that after infection, ethylene signalling is activated together with JA signalling in *N. attenuata* plants and this subsequently regulates scopoletin biosynthesis and plant resistance.

Keywords: ACO, EIN2, ETR1, phytoalexin, plant resistance, scopoletin

Introduction

Plant pathogens are usually divided into biotrophs, necrotrophs and hemibiotrophs according to their lifestyle. Biotrophic pathogens derive nutrients from living plant tissues, while necrotrophs kill host tissues and feed on dead or dying cells. Hemibiotrophs display a biotrophic phase early during infection and a necrotrophic phase later. In response to pathogen attack, plants have evolved sophisticated defence mechanisms to sense and protect themselves by induction of a phytohormone blend. Although there are some exceptions, host plants activate the salicylic acid pathway to defend against biotrophic and hemibiotrophic pathogens, whereas jasmonic acid (JA) and ethylene pathways are induced against necrotrophic pathogens (Glazebrook, 2005; Mengiste, 2012).

Alternaria alternata (tobacco pathotype) is a necrotrophic fungus notorious for causing brown spot disease in *Nicotiana* plants, including cultivated tobacco, *Nicotiana tabacum* (LaMondia, 2001), and wild tobacco, *N. attenuata* (Schuck *et al.*, 2014; Sun *et al.*, 2014a). When *N. attenuata* leaves were infected by *A. alternata*, strong blue fluorescence was observed around the infection zones under UV light, and this fluorescence was mainly emitted by scopoletin and its β-glycoside form, scopolin (Sun *et al.*, 2014b). Phytoalexins are low molecular weight antimicrobial substances produced in plants after infection, and are suggested to play an important role in plant resistance against necrotrophs (Kliebenstein *et al.*, 2005). Scopoletin, a phenolic coumarin, was later demonstrated to be a phytoalexin playing a vital role in plant resistance against *A. alternata* in *N. attenuata* plants; scopoletin-deficient plants, generated by silencing its key biosynthetic gene, *Feruloyl-CoA 6′-hydroxylase 1* (*F6′H1*), were more susceptible to the fungus, and this chemical exhibited antifungal activity *in vitro* and *in vivo* (Sun *et al.*, 2014b).

Previous work has shown that the biosynthesis of scopoletin was completely dependent on JA signalling. *Altenaria alternata*-elicited blue fluorescence was completely abolished in JA-deficient plants silenced with the key JA biosynthetic gene, *Allene oxide cyclase* (irAOC plants), and in plants silenced with *COI1* (irCOI1 plants), the receptor of JA-Ile (Sun *et al.*, 2014b). Furthermore, both irAOC and irCOI1 plants were more susceptible to *A. alternata*. These data provided strong evidence of the role of JA in plant resistance to necrotrophs by regulating phytoalexin production.

Ethylene, a simple gaseous phytohormone, is a key regulator of not only plant growth and development but also plant responses to biotic and abiotic stresses (Chen *et al.*, 2005; Glazebrook, 2005; Mengiste, 2012; Larsen, 2015). The biosynthesis of ethylene comprises two steps, including the conversion of S-adenosyl-L-methionine

*E-mail: jinsongwu@mail.kib.ac.cn
†These authors contributed equally.

Published online 17 July 2016

(SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and oxidation of ACC to form ethylene. These two steps are catalysed by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Larsen, 2015). ACS and ACO are encoded by multigene families consisting of four members of each in *N. attenuata* (von Dahl *et al.*, 2007). In order to fine-tune the specific response in physiological processes, plants regulate different isoforms of ACS and ACO to cope with different developmental stages and stresses (Wang *et al.*, 2002).

Ethylene is perceived by a family of five functional overlap membrane receptors, including ETR1, ETR2, ERS1, ERS2 and EIN4 (Chen *et al.*, 2005). Loss of function of any single receptor has little effect on seedling growth (Hua & Meyerowitz, 1998), but ethylene-insensitive plants can be generated by constitutive over-expression of *Arabidopsis etr1-1* mutant genes (Ov-etr1 plants), such as Ov-etr1 plants in *N. attenuata* (von Dahl *et al.*, 2007). CTR1 is the next downstream component in the ethylene signalling pathway, repressing ethylene response when in air. Binding of ethylene with receptors inactivates CTR1, and as a result EIN2 is activated, and a downstream transcriptional cascade is initiated (Chen *et al.*, 2005). EIN2 plays a major role in the ethylene signalling pathway, as the *ein2* mutation leads to complete blocking of ethylene responses (Alonso *et al.*, 1999).

A functional ethylene signalling pathway is important for plant resistance against necrotrophic pathogens (Mengiste, 2012). The blocking of ethylene signalling by *ein2* resulted in enhanced susceptibility of *Arabidopsis* to *Botrytis cinerea* (Thomma *et al.*, 1999). However, whether ethylene signalling also plays a role in the *N. attenuata–A. alternata* interaction and whether it is also involved in the regulation of scopoletin biosynthesis are still unknown.

The present study investigated whether ethylene signalling was involved in the *N. attenuata–A. alternata* interaction, by blocking the ethylene pathway by silencing *NaACO*, a key enzyme gene in ethylene biosynthesis, by over-expression of the *Arabidopsis* mutant receptor gene *ETR1*, and by silencing *NaEIN2*. The susceptibility of these plants to *A. alternata* was examined and the effect on scopoletin levels was measured.

Materials and methods

Plant and fungal material

Seeds of the 31st generation of an inbred line of *N. attenuata* were used as the wildtype (WT) genotype. 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) and used as plants that were silenced in the expression of *Allene oxide cyclase* (*NaAOC*; the gene encoding the key enzyme of JA biosynthesis) and three *1-Aminocyclopropane-1-carboxylic acid oxidases* (*NaACO*s; the genes for ethylene biosynthesis), and over-expression of *Arabidopsis* ethylene receptor mutant *etr1-1*gene, respectively. Seed germination and plant growth were conducted as described by Krugel *et al.* (2002).

Alternaria alternata was grown and inoculated as described by Sun *et al.* (2014a).

Real-time PCR assay

The preparation of total RNA and cDNA was performed as described by Wu *et al.* (2013). Real-time PCR was performed 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 *Actin2* as an internal standard from the same sample. All primer information is listed in Table S1.

Quantification of scopoletin and JA

Scopoletin extraction and quantification were performed as described by Sun *et al.* (2014b). In brief, about 0.1 g leaf samples collected from the inoculation sites (about 1 cm^2) were ground into fine powder in liquid nitrogen, and then 1 mL of 70% methanol was added to each sample. After vortexing for 10 min and centrifugation at 15 000 ***g*** for 20 min, supernatants were obtained and then subjected to analysis by microplate reader and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS; Thermo Scientific TSQ Quantum Access MAX).

The blue fluorescence intensity was measured by a microplate reader (Tecan Infinite M200 PRO) at an excitation wavelength of 320 nm and an emission wavelength of 420 nm. As the blue fluorescence intensity elicited by *A. alternata* was mainly due to scopoletin and scopolin (Sun *et al.*, 2014b), the total level of scopoletin (including scopolin) was calculated by comparing the microplate reader data with a standard curve of scopoletin (Sigma), and verified by HPLC-MS/MS.

Jasmonate was extracted and quantified by LC-MS/MS as described by Wu *et al.* (2008).

Generation of VIGS NaEIN2 plants

From RNA sequencing data (GEO accession number GSE61574) of *A. alternata*-elicited source–sink transition leaves (node 0 leaves), a *NaEIN2* cDNA (accession number KU308381) was found, which showed 98% of nucleotide sequence similarity with *EIN2* in *Nicotiana sylvestris* (accession number XM_009786989).

A 338 bp fragment of the *NaEIN2* cDNA sequence amplified by primers Z121_F and Z122_R was cloned into pTV00 (Ratcliff *et al.*, 2001), and *Agrobacterium tumefaciens* GV3101 cells carrying this construct were combined with those having pBINTRA, and inoculated into 25-day-old *N. attenuata* leaves, generating *NaEIN2*-silenced plants (virus-induced gene silencing (VIGS) NaEIN2). To monitor the progress of VIGS, *Phytoene desaturase* (PDS) was also silenced to generate plants with visible bleaching of green tissues about 2–3 weeks after the inoculation (Saedler & Baldwin, 2004; Wu *et al.*, 2008). When the leaves of *PDS*-silenced plants began to bleach (plants started bolting), the youngest rosette leaves of VIGS NaEIN2 and empty vector-inoculated plants (EV plants) were selected for further

experiments, as source–sink transition leaves were hard to distinguish in this stage. Around 40 plants were inoculated with EV and VIGS NaEIN2 constructs, and five biological replicates per genotype were used for experiments. All VIGS experiments were repeated twice.

Results

Induction of transcripts of ethylene biosynthetic genes after *A. alternata* infection

Previously, it was shown that JA signalling is essential for *A. alternata*-elicited phytoalexin scopoletin biosynthesis and host resistance against this necrotrophic fungus (Sun *et al.*, 2014b). To investigate whether ethylene signalling was also involved, the expression levels of *NaACS*s and *NaACO*s were analysed, in source–sink transition leaves (node 0 leaves) of rosette stage plants at 1 day post-inoculation (dpi) by real-time PCR. As four different *NaACS* (*NaACS1*, *NaACS2*, *NaACS3a* and *NaACS3b*) and *NaACO* (*NaACO1*, *NaACO2a*, *NaACO2b* and *NaACO3*) genes exist in the *N. attenuata* genome (von Dahl *et al.*, 2007), gene-specific primers were designed individually for *NaACS1*, *NaACS2*, *NaACO1* and *NaACO3*, and for *NaACS3* (for both *NaACS3a* and *NaACS3b*) and *NaACO2* (for both *NaACO2a* and *NaACO2b*) because of their high sequence similarities. The results showed that all *NaACS*s and *NaACO*s were strongly up-regulated after infection except *NaACS2* (Fig. 1). In particular, transcript levels of *NaACS1*, *NaACO1* and *NaACO2* were increased by 50-, 5000- and 150-fold, respectively, when compared with mock treatments (Fig. 1). These results indicate that most of the ethylene biosynthetic genes were dramatically up-regulated after infection, and gave a clue that ethylene signalling was activated after fungal attack, and might be involved in the fungus-induced defence responses.

IrACO and Ov-etr1 plant susceptibility to *A. alternata*

To test the hypothesis that ethylene signalling was involved in the resistance against *A. alternata*, the lesion diameter was compared of node 0 and +3 leaves (fully expanded leaves with three phyllotaxic position older than the leaf at node 0 (Sun *et al.*, 2014a)) of WT and transgenic plants of irACO and Ov-etr1 generated previously by von Dahl *et al.* (2007). All *NaACO* genes were successfully silenced in irACO plants, and the *Arabidopsis* ethylene receptor mutant *etr1-1* gene was overexpressed in Ov-etr1 plants, thus generating ethylene deficient and insensitive plants (von Dahl *et al.*, 2007). The silencing efficiency of the individual *NaACO* genes was checked again in this study by real-time PCR, and the transcripts of all *NaACO* genes were dramatically reduced in irACO plants by around 90% when compared with WT plants after infection (Fig. S1), indicating all *NaACO* genes were successfully silenced in irACO plants.

When detached node 0 leaves of WT, irACO and Ov-etr1 plants were infected with *A. alternata*, bigger lesions of around 0.8–0.9 cm developed in both irACO and Ov-etr1 plants, compared to around 0.45 cm in WT (Fig. 2),

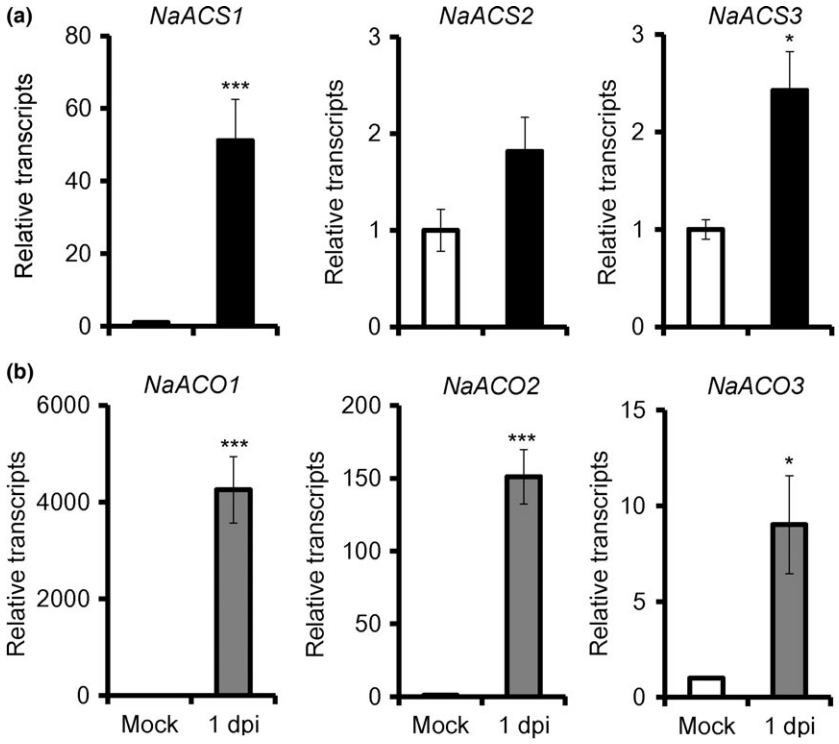

Figure 1 *Alternaria alternata*-elicited transcriptional levels of *NaACS*s and *NaACO*s. (a) Mean (±SE) *NaACS* transcripts were measured by real-time PCR in five replicates of node 0 leaves treated with mock or *A. alternata* at 1 day post-inoculation (dpi). (b) Mean (±SE) *NaACO* transcripts were measured by real-time PCR in five replicates of node 0 leaves treated with mock or *A. alternata* at 1 dpi. All transcriptional levels were normalized with a housekeeping gene *Actin2*. The asterisk indicates the level of significant difference between mock and infected leaves (Student's *t*-test: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$).

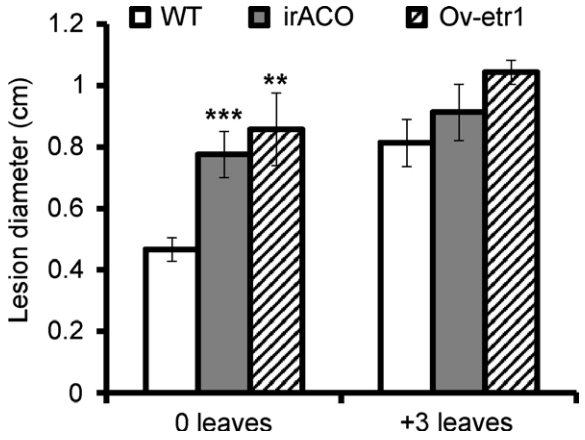

Figure 2 Mean (±SE) necrotic lesion diameters in five replicates of node 0 and +3 leaves of wildtype (WT), irACO (ethylene-deficient plants with silenced ethylene biosynthetic genes ACOs) and Ov-etr1plants (ethylene-insensitive plants with over-expression of *etr1* genes of *Arabidopsis*) after infection of *Alternaria alternata* at 5 days post-inoculation. The asterisk indicates the level of significant difference between WT and transgenic lines (Student's *t*-test: $^{**}P < 0.01$; $^{***}P < 0.005$).

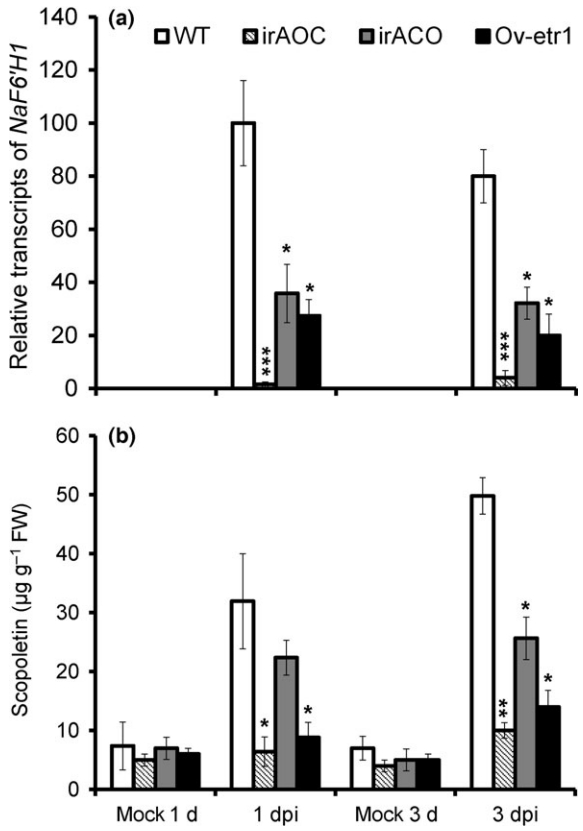

Figure 3 *Alternaria alternata*-induced *NaF6′H1* transcripts and scopoletin in wildtype (WT), irAOC (JA-deficient), irACO (ethylene-deficient) and Ov-etr1 (ethylene-insensitive) plants. (a) Mean (±SE) *NaF6′H1* transcripts were measured by real-time PCR in five replicates of node 0 leaves treated with mock or *A. alternata* at 1 and 3 days post-inoculation (dpi). (b) Mean (±SE) level of scopoletin as measured in six replicates of node 0 leaves at 1 and 3 dpi. The asterisk indicates the level of significant difference between WT and transgenic lines (Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$).

suggesting that the young source–sink transition leaves (node 0 leaves) in irACO and Ov-etr1 plants were more susceptible. However, the lesion diameters of +3 leaves of irACO and Ov-etr1 plants were slightly bigger, but not significantly so, than those of WT plants. These results strongly suggest that the ethylene signalling pathway plays an essential role in the resistance against *A. alternata* in *N. attenuata*, especially in young leaves.

Impaired production of scopoletin in irACO and Ov-etr1 plants

Scopoletin, a phenolic coumarin with blue fluorescence under UV light (Chong *et al.*, 2002; El Oirdi *et al.*, 2010; Gnonlonfin *et al.*, 2012), is a phytoalexin strongly accumulated around the infection zone of *N. attenuata* leaves, and whose induction is heavily dependent on JA signalling (Sun *et al.*, 2014b). However, whether the susceptibility of irACO and Ov-etr1 plants to *A. alternata* is also due to scopoletin deficiency is not known. Transcription levels were thus checked for *A. alternata*-elicited *Feruloyl-CoA 6′-hydroxylase 1* (*NaF6′H1*), the gene encoding the key enzyme for scopoletin biosynthesis, together with levels of scopoletin in WT, irACO and Ov-etr1 plants.

Consistent with previous findings (Sun *et al.*, 2014b), the transcriptional levels of *NaF6′H1* were strongly up-regulated in node 0 leaves of WT plants after infection, while JA-deficient irAOC plants (allene oxide cyclase gene silenced) accumulated only 1.5% and 4% of that of WT plants at 1 and 3 dpi, respectively (Fig. 3a). Interestingly, *NaF6′H1* transcripts were also significantly reduced by more than 50% in irACO and Ov-etr1 plants at both 1 and 3 dpi (Fig. 3a). Accordingly, *A. alternata*-elicited scopoletin levels were reduced in irACO and Ov-etr1 plants at 1 dpi, and the phenotype became more evident at 3 dpi, because their levels were only 51.5% and 28% of that of WT plants (Fig. 3b). In addition, scopoletin levels were also significantly decreased in +3 leaves when compared with those of node 0 leaves in irACO plants at 3 dpi (Fig. S2).

Taken together, the results indicate that irACO and Ov-etr1 plants were impaired in *A. alternata*-elicited scopoletin production.

NaEIN2 as an important ethylene signalling mediator

EIN2 is a very important transducer in the ethylene signalling cascade (Alonso *et al.*, 1999), located in the membrane of the endoplasmic reticulum (Bisson *et al.*, 2009). After receiving the signal from upstream, the EIN2 C terminus is cleaved and translocated to the nucleus to activate the downstream transcriptional cascade (Ju *et al.*, 2012). In *Arabidopsis*, the ethylene

signalling pathway is blocked in *EIN2*-mutated plants, and plants become sensitive to abiotic (Lei *et al.*, 2011; Niu & Guo, 2012) and biotic (Lu *et al.*, 2013) stress. To further confirm the role of ethylene signalling and to test if the downstream *NaEIN2* is also a scopoletin regulator after *A. alternata* infection in *N. attenuata*, *NaEIN2* was transiently silenced (90% transcriptionally silenced; Fig. 4a) by using the virus-induced gene silencing technique (to create VIGS-EIN2 plants), and the expression level of *NaF6′H1* and scopoletin production at 3 dpi was measured. *NaF6′H1* transcripts dropped dramatically by 76% in *NaEIN2*-silenced plants (VIGS NaEIN2 plants) compared to EV (empty vector) plants (Fig. 4b). Consistently, the production of scopoletin was also strongly decreased to 40% that of EV plants at 3 dpi (Fig. 4c). Moreover, the lesion diameters of VIGS EIN2 plants were 1.4-fold bigger than EV plants on average (Fig. 4d). Therefore, NaEIN2 is an important mediator of ethylene signalling, required for the fungus-elicited scopoletin biosynthesis and resistance.

Jasmonate and ethylene signalling and scopoletin biosynthesis

Previous work has shown that *A. alternata*-elicited scopoletin is completely dependent on JA production, because JA-deficient irAOC plants could not induce scopoletin levels at all after infection. In order to know whether the phenotypes of ethylene-deficient irACO plants were due to lowered JA production, levels of the fungus-elicited JA were checked in irACO and Ov-etr1 plants. Unexpectedly, JA levels were highly elicited in both irACO and Ov-etr1 plants at 1 dpi, with levels around threefold those of WT plants with the same treatments (Fig. 5a), and dropped to levels similar to WT plants at 3 dpi, indicating that the decreased *A. alternata*-induced scopoletin level of irACO and Ov-etr1 plants was not due to less JA production, and that ethylene signalling is required for the fungus-induced resistance, independent of JA.

The *A. alternata*-elicited transcripts of ethylene biosynthetic genes of *NaACO*s in JA-deficient irAOC plants were also checked. Results showed that the transcripts of *NaACO1* were significantly reduced in irAOC plants when compared with WT plants; however, transcripts of *NaACO2* and *NaACO3* were not affected (Fig. 5b), suggesting ethylene biosynthesis might be involved in the fungus-elicited scopoletin production in JA-deficient irAOC plants.

Discussion

Plant hormones are integrated into the plant immune response. In 2005, Glazebrook proposed that JA- and ethylene-signalling pathways affected resistance to necrotrophs in significant ways, whereas SA regulated resistance to biotrophs (Glazebrook, 2005). Indeed, when infected by the necrotrophic fungal pathogen *B. cinerea*,

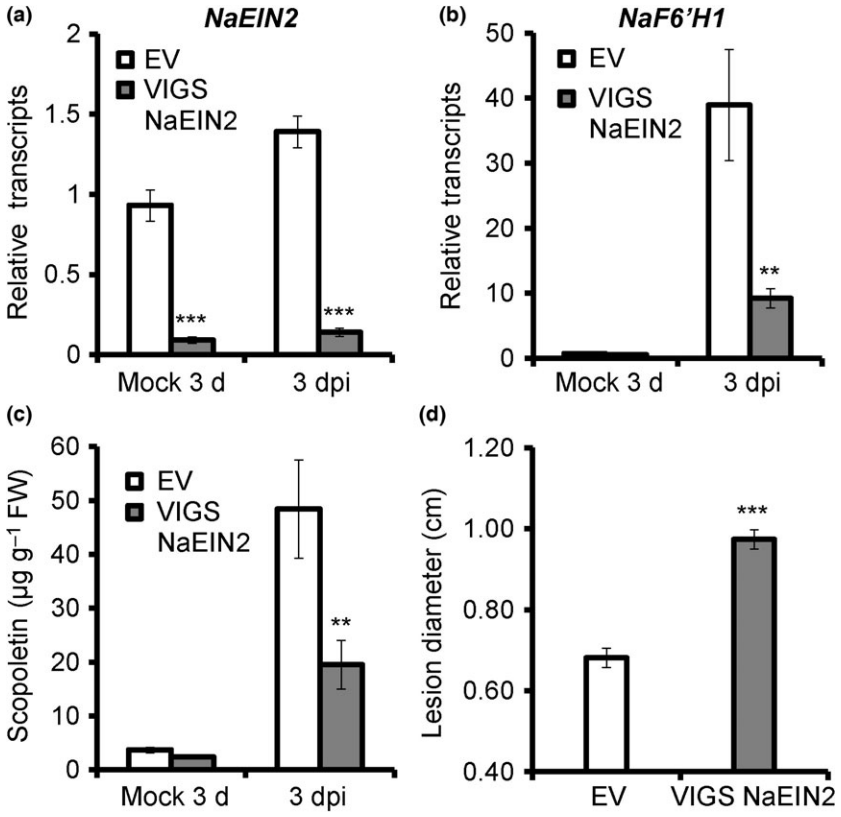

Figure 4 Reduced scopoletin production and resistance in plants silenced with *NaEIN2*. (a) Mean (±SE) relative *NaEIN2* transcript levels were measured by real-time PCR in four replicates of leaves of empty vector (EV) and VIGS NaEIN2 plants at 3 days post-inoculation (dpi). (b) Mean (±SE) relative *NaF6′H1* transcriptional levels were measured by real-time PCR in four replicates of leaves of EV and VIGS NaEIN2 plants at 3 dpi. (c) *Alternaria alternata*-elicited scopoletin in five replicates of leaves of EV and VIGS NaEIN2 plants at 3 dpi. (d) Mean (±SE) diameter of necrotic lesions in eight replicates of young leaves of EV and VIGS NaEIN2 plants infected with *A. alternata* for 7 days. The asterisk indicates the level of significant difference between EV and VIGS NaEIN2 plants (Student's *t*-test: $^{**}P < 0.01$; $^{***}P < 0.005$).

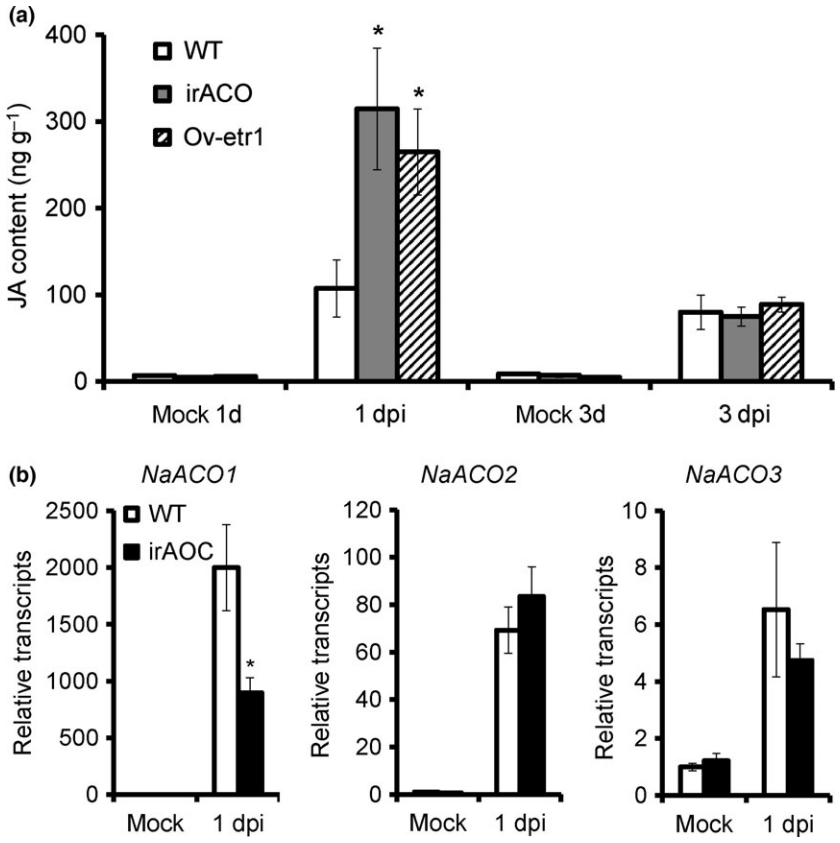

Figure 5 The levels of jasmonate (JA) in wildtype (WT), irACO and Ov-etr1 plants, and transcripts of *NaACO1*, *NaACO2* and *NaACO3* in WT and irAOC plants after infection. (a) Mean (±SE) level of JA as measured in five replicates of node 0 leaves at 1 and 3 days post-inoculation (dpi) WT, irACO and Ov-etr1 plants. (b) Mean (±SE) *NaACO1*, *NaACO2* and *NaACO3* transcripts were measured by real-time PCR in five replicates of node 0 leaves treated with mock or *Alternaria alternata* at 1 dpi in WT and irAOC plants. The asterisk indicates the level of significant difference between WT and transgenic lines (Student's *t*-test: $*P < 0.05$).

Arabidopsis plants activated the ethylene production and signalling pathway. These responses were important for resistance, because blocking of the ethylene signalling pathway by *ein2* resulted in enhanced susceptibility (Thomma *et al.*, 1999; Han *et al.*, 2010). However, the resistance of *Arabidopsis* to another necrotrophic fungal pathogen, *Alternaria brassicicola*, was not affected in the *ein2* mutant (van Wees *et al.*, 2003), suggesting that the contribution of ethylene signalling to the resistance against necrotrophs varied with pathogen species, although all were necrotrophic.

The necrotrophic fungal pathogen *A. alternata* f. sp. *lycopersici* causes diseases on tomato by producing AAL toxin (Akamatsu *et al.*, 1997). Ethylene signalling was induced by the infection of this fungus or treatments of AAL toxin, and conferred host susceptibility rather than resistance to this pathogen, probably by promotion of AAL toxin-induced cell death (Zhang *et al.*, 2011; Mase *et al.*, 2012; Jia *et al.*, 2013). However, this is not the case in the *N. attenuata*–*A. alternata* pathosystem here.

It has been proposed that AT toxin, a host cell death-inducer, is produced by the *A. alternata* tobacco pathotype after infection (Kohmoto *et al.*, 1981; Yakimova *et al.*, 2009; Cheng *et al.*, 2011). However, no detailed information is available about its chemical structure and mechanism of its induction of host cell death. The results here strongly indicate that ethylene signalling plays an important role in the resistance of wild tobacco against *A. alternata*, but not in AT toxin-induced host cell death, because (i) many ethylene biosynthetic genes of *N. attenuata* were up-regulated after infection (Fig. 1), and (ii) irACO, Ov-etr1 and VIGS NaEIN2 plants were more susceptible to the fungus (Figs 2 & 4). Thus, the results support Glazebrook's hypothesis that ethylene signalling plays an essential role in the resistance against necrotrophs.

At present, the mechanism underlying the ethylene-regulated resistance against necrotrophs is largely unknown. When *EIN2* was silenced in *N. benthamiana*, plants became more susceptible to *Phytophthora infestans*, a hemibiotrophic pathogen causing late blight disease of potato, and the expression of the key enzyme gene for capsidiol biosynthesis was abolished (Shibata *et al.*, 2010), indicating ethylene signalling might regulate host resistance through phytoalexin production. Previous work has shown that *N. attenuata* plants produce high amounts of the phytoalexin scopoletin, to defend against *A. alternata*, and this chemical defence is dependent on JA signalling (Sun *et al.*, 2014b). Now the data presented here provides new evidence that ethylene signalling is also important for fungus-elicited scopoletin production, as scopoletin levels dropped to 51.5%, 28.0% and 40.3% of WT plants in irACO, Ov-etr1 and VIGS NaEIN2 plants, respectively (Figs 3 & 4), while JA production was increased in irACO and Ov-etr1 plants (Fig. 5).

How scopoletin is regulated by ethylene is still not clear. The increased JA production in irACO and

Ov-etr1 plants at 1 dpi indicates that the decreased scopoletin level of those plants after infection is due to the blocking of ethylene signalling, but not JA; and ethylene signalling is required for scopoletin production independent of JA signalling, although plants might compensate by producing more JA. On the other hand, JA-deficient irAOC plants, which were unable to produce scopoletin, had WT levels of transcripts of *NaACO2* and *NaACO3*, and only transcripts of *NaACO1* were affected (Fig. 5), suggesting that ethylene production is only slightly reduced in irAOC plants, and that JA is the key signalling molecule regulating scopoletin. However, further efforts are still required to understand how JA and ethylene work together to regulate scopoletin biosynthesis and plant resistance.

In summary, the data strongly support the idea that in parallel to JA signalling, *N. attenuata* plants activate ethylene signalling to regulate plant resistance and scopoletin biosynthesis when infected by the necrotrophic fungal pathogen *A. alternata*.

Acknowledgements

The authors thank Professor Ian T. Baldwin (Max-Planck Institute for Chemical Ecology, Jena, Germany) for providing all the transgenic *N. attenuata* seeds and for discussion; and Biological Technology Open Platform of Kunming Institute of Botany (CAS) for greenhouse and instrument services. This project is supported by the Key Project of Applied Basic Research Programme of Yunnan (2014FA040), 100-Overseas-Top-Talents Recruitment plan of Yunnan, and Scientific Research Foundation for Returned Scholars to J.S.W. from the Ministry of Education of China.

References

Akamatsu H, Itoh Y, Kodama M, Otani H, Kohmoto K, 1997. AAL-toxin-deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. *Phytopathology* **87**, 967–72.

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR, 1999. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* **284**, 2148–52.

Bisson MM, Bleckmann A, Allekotte S, Groth G, 2009. EIN2, the central regulator of ethylene signalling, is localized at the ER membrane where it interacts with the ethylene receptor ETR1. *Biochemical Journal* **424**, 1–6.

Chen YF, Etheridge N, Schaller GE, 2005. Ethylene signal transduction. *Annals of Botany* **95**, 901–15.

Cheng DD, Jia YJ, Gao HY *et al.*, 2011. Characterization of the programmed cell death induced by metabolic products of *Alternaria alternata* in tobacco BY-2 cells. *Physiologia Plantarum* **141**, 117–29.

Chong J, Baltz R, Schmitt C, Beffa R, Fritig B, Saindrenan P, 2002. Downregulation of a pathogen-responsive tobacco UDP-Glc: phenylpropanoid glucosyltransferase reduces scopoletin glucoside accumulation, enhances oxidative stress, and weakens virus resistance. *The Plant Cell* **14**, 1093–107.

von Dahl CC, Winz RA, Halitschke R, Kuhnemann F, Gase K, Baldwin IT, 2007. Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *The Plant Journal* **51**, 293–307.

El Oirdi M, Trapani A, Bouarab K, 2010. The nature of tobacco resistance against *Botrytis cinerea* depends on the infection structures of the pathogen. *Environmental Microbiology* **12**, 239–53.

Glazebrook J, 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* **43**, 205–27.

Gnonlonfin GJB, Sanni A, Brimer L, 2012. Scopoletin – a coumarin phytoalexin with medicinal properties. *Critical Reviews in Plant Sciences* **31**, 47–56.

Han L, Li GJ, Yang KY *et al.*, 2010. Mitogen-activated protein kinase 3 and 6 regulate *Botrytis cinerea*-induced ethylene production in *Arabidopsis*. *The Plant Journal* **64**, 114–27.

Hua J, Meyerowitz EM, 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94**, 261–71.

Jia C, Zhang L, Liu L, Wang J, Li C, Wang Q, 2013. Multiple phytohormone signalling pathways modulate susceptibility of tomato plants to *Alternaria alternata* f. sp. *lycopersici*. *Journal of Experimental Botany* **64**, 637–50.

Ju C, Yoon GM, Shemansky JM *et al.*, 2012. CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 19486–91.

Kallenbach M, Bonaventure G, Gilardoni PA, Wissgott A, Baldwin IT, 2012. *Empoasca leafhoppers* attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. *Proceedings of the National Academy of Sciences of the United States of America* **109**, E1548–57.

Kliebenstein DJ, Rowe HC, Denby KJ, 2005. Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. *The Plant Journal* **44**, 25–36.

Kohmoto K, Hosotani K, Otani H, Nishimura S, 1981. Partial purification of host-specific toxin (AT) toxin produced by tobacco pathotype of *Alternaria alternata*. *Annals of the Phytopathological Society of Japan* **47**, 384.

Krugel T, Lim M, Gase K, Halitschke R, Baldwin IT, 2002. *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* **12**, 177–83.

LaMondia JA, 2001. Outbreak of brown spot of tobacco caused by *Alternaria alternata* in Connecticut and Massachusetts. *Plant Disease* **85**, 230.

Larsen PB, 2015. Mechanisms of ethylene biosynthesis and response in plants. *Essays in Biochemistry* **58**, 61–70.

Lei G, Shen M, Li ZG *et al.*, 2011. EIN2 regulates salt stress response and interacts with a MA3 domain-containing protein ECIP1 in *Arabidopsis*. *Plant, Cell & Environment* **34**, 1678–92.

Lu BB, Li XJ, Sun WW *et al.*, 2013. AtMYB44 regulates resistance to the green peach aphid and diamondback moth by activating EIN2-affected defences in Arabidopsis. *Plant Biology* **15**, 841–50.

Mase K, Mizuno T, Ishihama N *et al.*, 2012. Ethylene signaling pathway and MAPK cascades are required for AAL toxin-induced programmed cell death. *Molecular Plant–Microbe Interactions* **25**, 1015–25.

Mengiste T, 2012. Plant immunity to necrotrophs. *Annual Review of Phytopathology* **50**, 267–94.

Niu YH, Guo FQ, 2012. Nitric oxide regulates dark-induced leaf senescence through EIN2 in *Arabidopsis*. *Journal of Integrative Plant Biology* **54**, 516–25.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC, 2001. Technical advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal* **25**, 237–45.

Saedler R, Baldwin IT, 2004. Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in *Nicotiana attenuata*. *Journal of Experimental Botany* **55**, 151–7.

Schuck S, Weinhold A, Luu VT, Baldwin IT, 2014. Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*. *PLoS ONE* **9**, e102915.

Shibata Y, Kawakita K, Takemoto D, 2010. Age-related resistance of *Nicotiana benthamiana* against hemibiotrophic pathogen *Phytophthora infestans* requires both ethylene- and salicylic acid-mediated signaling pathways. *Molecular Plant–Microbe Interactions* **23**, 1130–42.

Sun H, Hu X, Ma J *et al.*, 2014a. Requirement of ABA signalling-mediated stomatal closure for resistance of wild tobacco to *Alternaria alternata*. *Plant Pathology* **63**, 1070–7.

Sun H, Wang L, Zhang B *et al.*, 2014b. Scopoletin is a phytoalexin against *Alternaria alternata* in wild tobacco dependent on jasmonate signalling. *Journal of Experimental Botany* **65**, 4305–15.

Thomma BP, Eggermont K, Tierens KF, Broekaert WF, 1999. Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiology* **121**, 1093–102.

Wang KLC, Li H, Ecker JR, 2002. Ethylene biosynthesis and signaling networks. *The Plant Cell* **14**, S131–51.

van Wees SC, Chang HS, Zhu T, Glazebrook J, 2003. Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. *Plant Physiology* **132**, 606–17.

Wu J, Wang L, Baldwin IT, 2008. Methyl jasmonate-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA? *Planta* **227**, 1161–8.

Wu J, Wang L, Wunsche H, Baldwin IT, 2013. Narboh D, a respiratory burst oxidase homolog in *Nicotiana attenuata*, is required for late defense responses after herbivore attack. *Journal of Integrative Plant Biology* **55**, 187–98.

Yakimova ET, Yordanova ZP, Slavov S, Kapchina-Toteva VM, Woltering EJ, 2009. *Alternaria alternata* AT toxin induces programmed cell death in tobacco. *Journal of Phytopathology* **157**, 592–601.

Zhang L, Jia C, Liu L, Zhang Z, Li C, Wang Q, 2011. The involvement of jasmonates and ethylene in *Alternaria alternata* f. sp. *lycopersici* toxin-induced tomato cell death. *Journal of Experimental Botany* **62**, 5405–18.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Transcriptional levels of *NaACO*s in wildtype (WT) and irACO plants. Mean (±SE) *NaACO1*, *NaACO2* and *NaACO3* transcripts were measured by real-time PCR in five replicates of node 0 leaves of WT and irACO plants treated with mock or *Alternaria alternata* at 1 day post-inoculation. The asterisk indicates the level of significant difference between WT and irACO plants (Student's *t*-test: $^{**}P < 0.01$).

Figure S2. *Alternaria alternata*-induced scopoletin in node 0 and +3 leaves of wildtype (WT), irAOC, and irACO plants. Mean (±SE) level of scopoletin as measured in six replicates of node 0 and +3 leaves at 3 days post-inoculation. The asterisk indicates the level of significant difference between node 0 and +3 leaves (Student's *t*-test: $^{*}P < 0.05$).

Table S1. All primers used in this study.