**Capsidiol, a defensive sesquiterpene produced by wild tobacco in response to
attack from the fungal pathogen *Alternaria alternata*, is regulated by an
ERF2-like transcription factor**

Na Song^{1,2†}, Lan Ma^{1†}, Weiguang Wang^{3†}, Huanhuan Sun¹, Lei Wang¹, Ian T.
Baldwin⁴ and Jinsong Wu^{1*}

¹Yunnan Key Laboratory for Wild Plant Resources, Kunming Institute of Botany,
Chinese Academy of Sciences, Lanhei Road 132, 650201, Kunming, China

²University of Chinese Academy of Science, Beijing 10049, China

³Key Laboratory of Chemistry in Ethnic Medicinal Resources of State Ethnic Affairs
Commission and Ministry of Education, Yunnan Minzu University, 650031, Kunming ,
China

⁴Department of Molecular Ecology, Max Planck Institute for Chemical Ecology,
07745 Jena, Germany

[†]These authors contributed equally

*** Corresponding author:**

Jinsong Wu
Kunming Institute of Botany, Chinese Academy of Sciences,
Lanhei Road 132, 650201 Kunming , P.R. China

Phone: 86-(0)871-65229552

Fax: 86-(0)871-65238769

Email: jinsongwu@mail.kib.ac.cn

1 **Date of submission:** March. 11 2019

2 **Number of tables:** 0

3 **Number of figures:**10 (Figures 1, 4, 6, 8 and 9 should be in color, both in print and
4 online)

5 **Total word count for the main body of the text: 5927**

6 Introduction: 503

7 Materials and Methods: 1845

8 Results: 2087

9 Discussion: 1091

10 Acknowledgements: 89

11 **Number of supplementary data:**

12 Figures: 3

13 Table: 3

14 **Running title:** Regulation of capsidiol-based defense by ERF2-like

15 **Highlight :** Our results demonstrate that capsidiol, a phytoalexin highly accumulated
16 in *Nicotiana attenuata* plants in response to *Alternaria alternata* attack, plays an
17 important role in pathogen resistance, and its biosynthesis is transcriptionally
18 regulated by an ERF2-like transcription factor.

19

20

21

22

23

24

25

Abstract

Capsidiol is a sesquiterpenoid phytoalexin produced in *Nicotiana* and *Capsicum* species in response to pathogen attack. Whether capsidiol plays a defensive role and how its biosynthesis is regulated in the wild tobacco *Nicotiana attenuata* when the plant is attacked by *Alternaria alternata* (tobacco pathotype), a notorious necrotrophic fungus causing brown spot disease, is unknown. Transcriptome analysis indicated that a metabolic switch to sesquiterpene biosynthesis occurred in young leaves of *N. attenuata* after *A. alternata* inoculation: many genes leading to sesquiterpene production were strongly up-regulated, including the capsidiol biosynthetic genes, *5-epi-aristolochene synthase* (*EAS*) and *5-epi-aristolochene hydroxylase* (*EAH*). Consistently, the level of capsidiol was increased dramatically in young leaves after fungal inoculation, from not detectable in mock control to 50.68 ± 3.10 $\mu\text{g/g}$ fresh leaves at 3 days post inoculation. Capsidiol-reduced or capsidiol-depleted plants, which were generated by silencing *EAHs* or *EASs* by virus-induced gene silencing, were more susceptible to the fungus. In addition, this sesquiterpene exhibited strong anti-fungal activities against *A. alternata* *in vitro* when purified from infected plants and applied to fungal growth. Furthermore, an ERF2-like transcription factor was found to positively regulate capsidiol production and plant resistance through the direct transactivation of a capsidiol biosynthetic gene *EAS12*. Taken together, our results demonstrate that capsidiol, a phytoalexin highly accumulated in *N. attenuata* plants in response to *A. alternata* infection, plays an important role in pathogen resistance independent of JA and ethylene signaling pathways, and its biosynthesis is transcriptionally regulated by an ERF2-like transcription factor.

Keywords: *5-epi-aristolochene hydroxylase* (*EAH*), *5-epi-aristolochene synthase* (*EAS*), *Nicotiana attenuata*, phytoalexin, ethylene responsive factor, virus induced gene silencing (VIGS).

Introduction

Plants are constantly attacked by a wide variety of microbial pathogens. In response, they activate a large number of intricate defense mechanisms, including the formation of reactive oxygen species, physical reinforcement of cell walls, production of phytohormones, antimicrobial proteins and metabolites (Glazebrook, 2005; Ahuja *et al.*, 2012; Mengiste, 2012). The class of small molecules known as phytoalexins is produced by plants *de novo* in response to pathogen attack, and is an important part of the plant defense repertoire (Ahuja *et al.*, 2012). In *Arabidopsis*, mutants impaired in the production of the phytoalexin camalexin are more susceptible to infection by necrotrophic fungi, such as *Alternaria brassicicola* (Nafisi *et al.*, 2007), *Botrytis cinerea* (Kliebenstein *et al.*, 2005) and *Plectosphaerella cucumerina* (Sanchez-Vallet *et al.*, 2010). In *Nicotiana* species, two phytoalexins have recently received attention: scopoletin (El Oirdi *et al.*, 2010; Sun *et al.*, 2014b) and capsidiol (Mialoundama *et al.*, 2009; Shibata *et al.*, 2010; Grosskinsky *et al.*, 2011; Shibata *et al.*, 2016).

Capsidiol has been proposed to be an important ‘chemical weapon’ against pathogens in *Nicotiana* species. This bicyclic sesquiterpene is produced via cyclization of farnesyl pyrophosphate (FPP) to 5-*epi*-aristolochene by 5-*epi*-aristolochene synthase (EAS), followed by two hydroxylation reactions catalyzed by 5-*epi*-aristolochene dihydroxylase (EAH) (Facchini and Chappell, 1992; Ralston *et al.*, 2001). Capsidiol exhibits toxicity towards many pathogens, including *Phytophthora capsici* and *B. cinerea* (Stoessl *et al.*, 1972; Ward *et al.*, 1974). Recently, molecular evidence also supports its role in non-host resistance against *P. infestans* in *N. benthamiana*, as *NbEAS*- or *NbEAH*-silenced plants were highly susceptible (Shibata *et al.*, 2010).

Ethylene response factors (ERFs) are transcription factors that play crucial roles in plant immunity (Huang *et al.*, 2016). In *Arabidopsis*, several ERFs have been identified as important regulators in *Botrytis* resistance, such as ORA59, ERF1 and RAP2.2 (Solano *et al.*, 1998; Berrocal-Lobo *et al.*, 2002; Pre *et al.*, 2008; Zhao *et al.*, 2012). Most ERFs are able to bind specifically to DNA sequences containing a GCC (GCC box) and/or a dehydration-responsive element/C-repeat (DRE/CRT box,

A/GCCGAC) (Hao *et al.*, 1998; Hao *et al.*, 2002). However, the ERF RAP2.2 binds the consensus sequence ATCTA in the promoter region of *phytoene synthase* and *phytoene desaturase* to regulate carotenoid biosynthesis (Welsch *et al.*, 2007).

The necrotrophic fungal pathogen *Alternaria alternata* (tobacco pathotype) causes brown spot disease in *Nicotiana tabacum* (LaMondia, 2001) and many other *Nicotiana* species, including the wild tobacco *Nicotiana attenuata* (Schuck *et al.*, 2014). Inoculation with *A. alternata* elicits the activation of both jasmonate and ethylene signaling pathways in *N. attenuata* plants, which subsequently lead to the accumulation of the phytoalexins, scopoletin and its glycoside form, scopolin (Sun *et al.*, 2014b; Li and Wu, 2016; Sun *et al.*, 2017). Currently it is not known if fungus-inoculated *N. attenuata* plants produce other phytoalexins, such as capsidiol. If yes, how is its biosynthesis transcriptionally regulated?

In this study, capsidiol was identified and confirmed to be an important phytoalexin produced in *N. attenuata* when challenged by *A. alternata*, and its regulation by an ERF2-like transcription factor was investigated in detail.

Materials and Methods

Plant and fungal materials

Seeds of the 31st generation of an inbred line of *Nicotiana attenuata* were used as the wild-type (WT) genotype. Ethylene deficient and insensitive (irACO and Ov-etr1, respectively), and jasmonate deficient (irAOC) *N. attenuata* plants were generated previously (von Dahl *et al.*, 2007; Kallenbach *et al.*, 2012). Seed germination and plant growth were conducted as described by Krügel *et al.*, (2002).

Alternaria alternata were grown and inoculated into leaves as described by Sun *et al.*, (2014a).

RNA-seq data processing and analysis

Source-sink transition leaves of rosette-staged WT plants (35-day-old) were detached and inoculated with *A. alternata* for 1 d, when only a few of fungal hyphae had penetrated into leaf tissues (Sun *et al.*, 2014a). Total RNA of 3 biological

replicates mock (WT_0L_M, with sample names S716, S717 and S719) or inoculated leaf samples (WT_0L_Inf, with sample names S20, S722 and S726) were isolated with TRIzol reagent (Invitrogen). RNA sequencing was conducted by Shanghai OE-Biotech (<http://www.oebiotech.com/>) with Illumina Hiseq 2000.

Sequencing was performed at 8 G depth, and mapped to the *N. attenuata* reference genome sequence. The relative abundance of the transcripts was measured with the FPKM (RPKM) method, which measures the transcripts abundance as RPKM (Reads Per Kilobase of exon model per Million mapped reads). The differential expressions between mock and inoculated 1 dpi samples with a cutoff of two-fold change, and its significance, were calculated.

Generation of *NaEAS*- and *NaEAH*-silenced VIGS plants

A 579 bp *NaEAS* cDNA fragment amplified with primers (Z003_F and Z004_R, Supplementary Table S3) and a 424 bp fragment of *NaEAH* cloned with primers (Z047_F and Z048_R, Supplementary Table S3) were individually inserted into pTV00 (Ratcliff *et al.* 2001) in reverse orientations. *Agrobacterium tumefaciens* (strain GV3101) harboring these constructs were mixed with the strain with pBINTRA and inoculated into *N. attenuata* leaves generating *NaEAS*s- and *NaEAH*s-silenced plants (VIGS *NaEAS*s and VIGS *NaEAH*s). The *A. tumefaciens*-mediated transformation procedure was performed as described previously (Saedler and Baldwin, 2004). To monitor the progress of VIGS, *phytoene desaturase* (*PDS*) was also silenced. Silencing *PDS* results in the visible bleaching of green tissues (Saedler and Baldwin, 2004; Wu *et al.*, 2008) about 2 to 3 weeks after the inoculation. When the leaves of *PDS*-silenced plants began to bleach, the young leaves of VIGS plants and empty vector-inoculated plants (EV plants) were selected for further experiments. Around 20 plants were inoculated with each construct, and usually 10 biological replicates per construct exhibiting efficient silencing were used for each experiment, and all VIGS experiments were repeated twice.

Purification and quantification of capsidiol in *N. attenuata* after infection

1 Around 500 g leaves which had been inoculated with *A. alternata* for 3 d, were
2 collected for capsidiol extraction. Leaves were twice extracted with 70 % acetone (2 L)
3 at room temperature. The solvent was evaporated and suspended in water, and then
4 extracted with ethyl acetate. The ethyl acetate-soluble fraction (10 g) was decolorized
5 on MCI gel (www.gls.co.jp) with methanol: H₂O (90:10) to obtain a yellow gum (7 g),
6 which was subsequently purified by silica gel column with a chloroform: acetone
7 gradient system (from 10:0, 9:1, 8:2, 7:3, 6:4, to 1:1) to yield six main fractions (A–F).
8 Fraction B (chloroform: acetone, 9:1; 2 g) was subjected to repeated chromatography
9 over silica gel (petroleum ether: acetone, from 30:1 to 1:1) to yield fractions B1-B4.
10 Fraction B3 (petroleum ether: acetone, 10:1) was separated further by RP-18 column
11 (acetonitrile: H₂O, 30:70). The obtained crude capsidiol was further purified by
12 semi-preparative HPLC (3 mL/min, UV detection at $\lambda_{\max} = 202$ nm, acetonitrile: H₂O,
13 40:60; ZORBAX SB-C18 column (5 μ m, 9.4 $\times$ 250 mm, Agilent 1200, USA) to yield
14 capsidiol (30 mg, > 99.5 % purity). The purified capsidiol showed the same
15 characteristic NMR data and HPLC retention times as compared with the authentic
16 standard provided by Prof. Joe Chappell (University of Kentucky, USA).

17 *A. alternata*-elicited capsidiol levels were determined by HPLC by reference to
18 the authentic capsidiol standard. Each leaf was inoculated with four agar plugs with
19 fungal mycelium, and a 1.5 $\times$ 1.5 cm² area of the leaf lamina was harvested at 1 or 3
20 dpi (around 200 mg fresh mass). Samples were grounded in liquid nitrogen and twice
21 extracted with 1 mL dichloromethane. 2 mL extracts were dried in a SpeedVac
22 concentrator (Eppendorf), and finally dissolved in 1 mL methanol for HPLC. At a
23 flow rate of 1 mL/min, 10 μ L of each sample was injected onto a ZORBAX SB-C18
24 column (5 μ m, 4.6 $\times$ 250 mm) (Agilent 1260, USA). The mobile phase was composed
25 of solvent A (water) and solvent B (acetonitrile) was used in a isocratic elution (40%
26 of B). Capsidiol was detected at 202 nm, with a retention time of 10.38 min. The
27 standard capsidiol was dissolved in methanol at six concentrations (6.25, 12.5, 25, 50,
28 100, and 200 μ g/mL) to create an external standard curve which was used to
29 calculated fungal-induced capsidiol levels.

30

Bioassays for the inhibition of *A. alternata* growth by capsidiol *in vitro*

The inhibition of *A. alternata* mycelium growth by capsidiol *in vitro* was tested in Petri dishes by sub-culturing a 3 mm diameter mycelium plug on PDA medium containing various concentrations of capsidiol for 6 days in the dark at 25°C. Twenty mg capsidiol was dissolved in 10 mL methanol, and added to the PDA media at final concentrations of 0, 50, 100 and 200 µg/mL. PDA plates with 1% methanol were served as controls. Photos were taken every two days, and the area of mycelium growth was calculated with ImageJ (<http://imagej.nih.gov/ij/>).

Quantification of scopoletin and scopolin

Around 0.2 g leaf samples at 6 dpi of EV, VIGS NaEAS and VIGS NaEAH plants were harvested and ground to fine powder in liquid nitrogen. The levels of scopoletin and scopolin were determined by HPLC-MS/MS as described in Sun *et al.*, (2014b).

Real-Time PCR

Total RNA was extracted from a 1.5 x 1.5 cm² area of leaf lamina which encompassed the inoculation site with TRI reagent (Invitrogen). For each treatment, 4-5 replicate biological samples were collected. cDNA was synthesized from 500 ng total RNA with reverse transcriptases (Thermo Scientific). 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 as described (Wu *et al.*, 2013). 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 transcript levels of unknown samples were calculated according to the standard curve. Finally, the 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 *NaActin 2* as an internal standard from the same sample. The transcript abundance of *NaActin 2* was not altered in leaves inoculated with *A. alternata* at 1 and 3 dpi (Xu *et al.*, 2018). All primers were listed in Supplementary

1 Table S3.

2

3 **Transient expression assays**

4 To determine the subcellular localization of NaERF2-like, a 35S::ERF2-eGFP
5 construct was used for transient expression in *N. attenuata* protoplasts. The
6 Full-length coding sequence of *NaERF2-like* was amplified by primers Z141_F and
7 Z142_R (Supplementary Table S3) and inserted into vector pM999 via *Sac* I and *Xho*
8 I. The method of protoplast isolation and transient transformation was adopted from
9 (Yoo *et al.*, 2007) with some modifications. In brief, mesophyll protoplasts were
10 isolated from source-sink transition leaves, and twenty microgram of plasmids was
11 transfected into 2×10^5 protoplasts with polyethylene glycol (PEG) solution (0.4 g/ml
12 PEG 4000, 0.2 M mannitol, 0.1M CaCl₂). Transformed cells were cultured in solution
13 (4 mM MES, 0.5 M mannitol, 20 mM KCl) for 18 h in dark and images were obtained
14 with a fluorescent microscope (Leica DM5500 B) with excitation at 488 nm for GFP
15 signal.

16 For transient transactivation assay, the promoter region of *NaEAS12* (-1671 to -1;
17 numbered from the first ATG) and firefly luciferase (LUC) were amplified and cloned
18 into pCAMBIA1301. Next, the PCR fragment including promoter region of *NaEAS12*,
19 *LUC*, and Nos terminator was subcloned into pMD18 via *Hind* III and *Sac* I to reduce
20 the size of the vector and increase the transformation efficiency. Similarly, 35S::
21 NaERF2-like-2HA-eGFP with Nos terminator was also subcloned into pMD18 vector.
22 *N. attenuata* protoplasts were prepared as describe above, and transformed with both
23 NaEAS12_{Promoter}::LUC and 35S:: NaERF2-like-2HA-eGFP, or with
24 NaEAS12_{Promoter}::LUC and 35S:: 2HA-eGFP as control. After transformation, the
25 protoplasts were subjected to RNA extraction with a PrimeScript™ RT reagent Kit
26 with gDNA Eraser and then gene expression assay. All the experiments were repeated
27 twice with similar results.

28

29 **Yeast-one-hybrid assay**

30 The Matchmaker yeast-one-hybrid system (Clontech) was used to test the binding

1 of NaERF2-like and the NaEAS12 promoter *in vitro* according to the user manual.
2 The promoter region of *NaEAS12* (pEAS12-b; -926 to -699; numbered from the first
3 ATG) which contained the candidate binding motif of EM13
4 (5'-tagattATCTaattctact-3'), was inserted into pAbAi vector with *Hind* III and *Xho* I.
5 The bait construct was linearized and integrated into the genome of yeast strain
6 Y1HGold. The full-length coding sequence of *NaERF2-like* was introduced into
7 pGADT7 AD vector via *Cla* I and *Xho* I, and then the construct was transformed into
8 the yeast cells containing the bait. The positive clones were analyzed on SD/-His/-Leu
9 medium supplied with 200 ng/mL (final conc.) 3-amino-1, 2, 4-triazole (3-AT).
10 Y1HGold [pGADT7/ pEAS12-b-AbAi] was used as negative control, and Y1HGold
11 [pGADT7 Rec-p53/p53-AbAi] was used as positive control.

12

13 Electromobillity shift assays (EMSA)

14 The full-length coding sequence of *NaERF2-like* was cloned in frame into the
15 *EcoR* I-*Xho* I sites of the pET28a (+), His-NaERF2-like were expressed and purified
16 with Ni-NTA agarose (QIAGEN). Biotin labeled probe EM13
17 (5'-tagattATCTaattctact-3') and mutant probe (5'-tagattAATTaattctact-3') were
18 synthesized from Sangon Biotech (Shanghai). The detection of the binding of the
19 recombinant protein and the probes (300 ng of recombinant protein and 30 ng labeled
20 probe) were carried out with a chemiluminescent EMSA kit (Beyotime Biotechnology)
21 according to the protocol suggested by the manufacture.

22

23 Generation of Ov-NaERF2-like plants and chromatin immune-precipitation 24 assay

25 The full-length cDNA of NaERF2-like with two HA flags was cloned into
26 pCAMBIA1301 vector after 35S promoter via in-fusion technique (Clontech). *N.*
27 *attenuata* plants (31st generation of an inbred line) were transformed by
28 *Agrobacterium tumefaciens* with this construct according to Krügel *et al.*, (2002). T1
29 seeds were screened for single T-DNA inserts (1:3 segregation of hygromycin
30 resistance), and two lines of T2 (Ov-NaERF2-like line 1 and 2) with HA signals

(Supplementary Fig. S2) were selected and used in this study.

Chromatin immunoprecipitation was performed with EpiQuik Plant ChIP Kit (EPIGENTEK) according to its user manual. The source-sink transition leaves of Ov-NaERF2-like line 2 at 1 dpi (1 g) were used for ChIP assays. A ChIP grade anti-HA antibody (Abcam) was used to immunoprecipitate the protein-DNA complex, and the precipitated DNA was further purified for real time PCR by using primer sets from *NaEAS12* promoter (5' - CACTTTAACCCCGGGTAACT-3' and 5' - CACTTCTCAGATTCTCCAGTTTGG-3') and *NaActin 2* gene (5' - GGTCGTACCACCGGTATTGTG-3' and 5' - GTCAAGACGGAGAATGGCATG-3') for negative control. The ChIP experiments were performed twice with similar results. Chromatins which were precipitated without antibody served as the negative controls.

Results

Transcriptome analysis reveals the strong regulation of sesquiterpene

biosynthetic genes in *N. attenuata* after *A. alternata* inoculation

Previously, we have demonstrated that, in response to *A. alternata* inoculation, *N. attenuata* plants activate both jasmonate and ethylene signaling pathways to regulate the biosynthesis of the phytoalexins scopoletin and its β -glycoside form, scopolin (Sun *et al.*, 2014b; Li and Wu, 2016; Sun *et al.*, 2017). As *feruloyl-CoA 6'-hydroxylase 1* (*NaF6'H1*), the key enzyme gene for scopoletin biosynthesis, was highly elicited at 1 day post inoculation (1 dpi) when the infection was at early stage and only a few of fungal hyphae had been observed to penetrate into leaf tissues via stomata (Sun *et al.*, 2014a), transcriptome analysis was performed in *A. alternata*-inoculated *N. attenuata* leaves at 1 dpi to identify secondary metabolites which could potentially act similarly to scopoletin. Notably, a set of genes involved in terpene synthesis were strongly up-regulated, including *thiolase*, *HMG-CoA synthase*, *HMG-CoA reductase*, *mevalonate kinase*, *phosphomevalonate kinase*, *diphosphomevalonate decarboxylase* (*MVPP decarboxylase*), *isopentenyl-diphosphate delta-isomerase*, *FPP synthase*, the capsidiol biosynthetic genes *5-epi-aristolochene synthases* (*EASs*) and *5-epi-aristolochene*

1 *1,3-dihydroxylases (EAHs)*, and the solavetivone biosynthetic genes *premnaspirodiene*
 2 *oxygenase* and *premnaspirodiene synthase* (Fig. 1 and Supplementary Table S1).
 3 Meanwhile the *squalene synthase* involved in triterpene biosynthesis was
 4 down-regulated (Fig. 1 and Supplementary Table S1). These results strongly indicate
 5 that sesquiterpene biosynthetic pathway is activated during the inoculation of *A.*
 6 *alternata*.

7 To confirm the regulation of terpene biosynthesis genes in response to *A.*
 8 *alternata*, we performed quantitative real time PCR on *N. attenuata* samples collected
 9 at 1 dpi. Compared with mock infection controls, the transcripts of *MVPP*
 10 *decarboxylase*, *HMG-CoA reductase*, *FPP synthase* at 1 dpi were increased to 322-,
 11 53-, and 14-fold respectively, while the transcripts of *squalene synthase* were reduced
 12 by 75% (Fig. 1). More importantly, transcripts of the capsidiol biosynthetic genes
 13 *NaEASs* and *NaEAHs*, both of them were encoded by a multi-gene family
 14 (Supplementary Table S1) and thus were detected by primers designed at the
 15 consensus region, increased to 368- and 40-fold compared to control (Fig. 1). These
 16 data fully confirmed the transcriptomics result of strong activation of the
 17 sesquiterpene biosynthetic pathway in *N. attenuata* after inoculation.

18 19 **Accumulation of capsidiol in *N. attenuata* in response to *A. alternata* inoculation**

20 Since *NaEAS* and *NaEAH* are key genes in the capsidiol biosynthesis pathway,
 21 we expected to see an increase in capsidiol production after *A. alternata* inoculation.
 22 The levels of capsidiol at 1 and 3 dpi in the young source-sink transition leaves (0
 23 leaves) and mature leaves (+3 leaves) were determined by a high-performance liquid
 24 chromatography (HPLC). The +3 leaves are three phyllotaxic positions older than 0
 25 leaves, and are more susceptible to *A. alternata* (Sun *et al.*, 2014a). Our results
 26 indicated that capsidiol was not detectable in the 0 leaves of mock control, but its
 27 level was increased to 4.45 ± 0.72 $\mu\text{g/g}$ fresh leaves in 0 leaves at 1 dpi, and to 50.68
 28 ± 3.10 $\mu\text{g/g}$ at 3 dpi (Fig. 2). Interestingly, this compound was also detected in the
 29 susceptible +3 leaves, but its level was only about one-third of that of 0 leaves, with
 30 1.66 ± 1.00 $\mu\text{g/g}$ fresh leaves at 1 dpi, and 14.54 ± 4.39 $\mu\text{g/g}$ at 3 dpi (Fig. 2). These

results indicate that 1) capsidiol is highly elicited in *N. attenuata* leaves after inoculation; 2) the lower accumulations of capsidiol in mature leaves may account for their susceptibility to the fungus.

Capsidiol accumulation is essential for *A. alternata* resistance in *N. attenuata*

To further evaluate the role of capsidiol in *N. attenuata* resistance against *A. alternata*, we silenced *NaEASs* and *NaEAHs* separately with their conserved sequences, as both are encoded by members of a multi-gene family. Compared with mock controls, transcripts of *NaEAHs* were dramatically induced in young leaves of *N. attenuata* plants transformed with empty vector (EV) at 2 dpi; however, plants transformed with the *NaEAHs*-silencing construct (VIGS *NaEAHs*) showed a 92% reduction in *NaEAHs* transcripts compared to EV plants with the same treatments, indicating effective silencing of the *NaEAHs* (Fig. 3A). We also investigated capsidiol level in EV and VIGS *NaEAHs* plants at 3 dpi. Capsidiol levels at $57.72 \pm 5.88 \mu\text{g/g}$ fresh leaves were detected in EV plants, while only $8.99 \pm 1.56 \mu\text{g/g}$ fresh leaves were found in VIGS *NaEAHs* plants (Fig. 3B).

Because a small amount of capsidiol was still present in VIGS *NaEAHs* plants, we attempted to generate additional capsidiol-depleted *N. attenuata* plants by silencing *NaEASs*. *NaEASs* expression was successfully silenced, as only 7% of the transcripts of *NaEASs* were detected at 2 dpi in VIGS *NaEASs* plants compared with EV plants (Fig. 3A). More importantly, the *A. alternata*-elicited capsidiol levels at 3 dpi was abolished in VIGS *NaEASs* plants, with only 0.2 % of the levels detected in EV plants, which were comparable to the levels quantified in the mock controls of EV plants (Fig. 3B). From these data, we infer that *NaEASs* genes are crucial for *A. alternata*-elicited capsidiol production.

To test whether capsidiol-reduced or -depleted plants are more susceptible to *A. alternata*, young leaves of EV, VIGS *NaEAHs*, and VIGS *NaEASs* plants were inoculated with the fungus. Two independent VIGS experiments showed significantly increased lesion diameters in VIGS *NaEAHs* (121% of EV plants) and VIGS *NaEASs* plants (131% of EV plants) (Fig. 3C). Meanwhile, we did not observe changes in

scopoletin and scopolin, two important phytoalexins involved in *A. alternata* resistance (Fig. 3B). These results strongly indicate that capsidiol plays an important role in defending against *A. alternata*.

Capsidiol exhibits anti-fungal activity against *A. alternata* *in vitro*

To test whether capsidiol has a direct impact on fungal growth or not, we purified 30 mg capsidiol from 500 g of *A. alternata*-inoculated leaves, and applied this compound in various concentrations to the growth medium to evaluate its inhibition activity of fungal growth *in vitro*. The fungi were grown on PDA plates containing with 50 µg/mL or 100 µg/mL capsidiol, and we observed fungal growth was reduced to 56.6% or 43.8% of that of controls. Application of 200 µg/mL capsidiol resulted in further reduction in growth to 37.1% of control (Fig. 4A, B). These results suggest that capsidiol at a concentration observed *in planta* has a direct impact on the fungal growth *in vitro*.

***A. alternata*-induced capsidiol accumulation is not dependent on JA and ethylene signaling**

JA and ethylene signaling pathways are crucial for phytoalexin scopoletin biosynthesis. To investigate the roles of these two signaling pathways in capsidiol biosynthesis, we measured the levels of capsidiol and transcripts of *NaEASs* and *NaEAHs* after *A. alternata* inoculation in WT, JA-deficient (*irAOC*), ethylene-deficient (*irACO*), and ethylene-insensitive (*Ov-etr1*) plants generated previously (Kallenbach *et al.*, 2012; von Dahl *et al.*, 2007). We found that the induction levels of *NaEASs* and *NaEAHs* by *A. alternata* were similar in WT, *irAOC*, *irACO* and *Ov-etr1* plants at both 1 and 3 dpi (Fig. 5A, B). In addition, no significant differences of capsidiol production were observed in WT, *irAOC*, *irACO* and *Ov-etr1* plants at 3 dpi (Fig. 5C). Thus, our data indicated that *A. alternata*-induced capsidiol accumulation is not dependent on JA and ethylene signaling.

NaERF2-like, a transcription factor highly induced in young leaves, is required

to mount a capsidiol-based defense

Since we observed increases in both capsidiol production as well as *NaEASs* and *NaEAHs* transcripts in *N. attenuata* leaves after fungal inoculation, we hypothesized that the transcription factors regulating *NaEASs* and *NaEAHs* expression were also increased. Thus, we silenced the expression of the 6 fungus-elicited transcription factor genes with the most abundant transcripts after fungal inoculation (Supplementary Table S2), including *ethylene-responsive transcription factor ABR1-like* (*NaERF ABR1-like*; gene accession number : XM_019374371), *zinc finger protein ZAT12-like* (*NaZAT12*; gene accession number: XM_019368773.1), *probable WRKY transcription factor 40* (*NaWRKY40*; gene accession number: XM_019402562.1), *probable WRKY transcription factor 43* (*NaWRKY43*; gene accession number: XM_019375046.1), *probable WRKY transcription factor 61* (*NaWRKY61*; gene accession number: XM_019371308.1), *ethylene-responsive transcription factor 2-like* (*NaERF2-like*; gene accession number: XM_019399671.1), to identify the regulator(s) responsible for capsidiol biosynthesis. *A. alternata*-elicited *NaEAS12* was not altered in plants silenced with *NaERF ABR1-like*, *NaZAT12*, *NaWRKY40*, *NaWRKY43*, or *NaWRKY61* (Supplementary Fig. S1).

NaERF2-like (Gene accession number: XM_019399671.1), one of the top 6 transcription factors strongly up-regulated in response to fungal inoculation in our transcriptome analysis, was highly induced in *N. attenuata* 0 leaves at both 1 and 3 dpi (Fig. 6A; Supplementary Table S2). The *NaERF2-like* protein exhibited nuclear localization in the protoplast of *N. attenuata* when its eGFP fusion protein was driven by a constitutive 35S promoter (Fig. 6B).

To investigate the role of the *NaERF2-like* transcription factor in capsidiol biosynthesis in detail, we silenced the gene by VIGS, and then measured the levels of capsidiol and transcripts of *NaEASs* and *NaEAHs* after fungal inoculation. *NaERF2-like* transcripts were highly elicited at 2 dpi in EV plants; in contrast, VIGS *NaERF2-like* plants showed an 87% reduction in *NaERF2-like* transcripts after the same treatment (Fig. 7A). Compared to EV plants at 2 dpi, the transcripts of *NaEASs*

and *NaEAHs* in VIGS *NaERF2*-like plants were reduced by 75% and 62%, respectively (Fig. 7B, C). As expected, *A. alternata*-induced capsidiol level in VIGS *NaERF2*-like plants at 3 dpi was reduced by 68% when compared with EV plants (Fig. 7E). However, the fungus-elicited transcriptional levels of *NaF6'H1* were not affected in VIGS *NaERF2*-like plants (Fig. 7D). These results indicate that silencing *NaERF2-like* substantially decreases *A. alternata*-elicited transcription of *NaEASs* and *NaEAHs*, and consequently, capsidiol level, without affecting scopoletin-based defense.

In addition, silencing *NaERF2-like* led to plants more susceptible to *A. alternata*, as significantly larger lesions were observed in VIGS *NaERF2*-like plants at 6 dpi in two independent VIGS experiments (Fig. 7F).

NaERF2-like* directly regulates the capsidiol biosynthetic gene *NaEAS12

Next, we explored the mechanism by which *NaERF2-like* regulates capsidiol-based resistance. We hypothesized that *NaERF2-like* might directly regulate genes in the capsidiol biosynthetic pathway. Since *NaEAS12* expression was greatly reduced in *NaERF2-like*-silenced plants (Fig. 8A), we selected this gene to test whether its promoter could be directly activated by *NaERF2-like*.

Several lines of evidence were consistent with the idea that *NaERF2-like* directly regulates the capsidiol biosynthetic gene *NaEAS12*. When the *NaERF2-like* gene was over-expressed in the protoplasts of *N. attenuata*, *luciferase* gene (*LUC*) driven by the *NaEAS12* promoter showed a 5.5-fold increase in expression (Figure 8B), indicating that the over-expression of *NaERF2-like* enhanced the transcriptional activity of *NaEAS12* promoter. From the promoter region of *NaEAS12*, an ATCTA motif, previously shown to be the binding site of RAP2.2 in *Arabidopsis* (Welsch *et al.*, 2007), was identified and confirmed as the binding site for the ERF2-like by yeast-one-hybrid, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation-based qPCR (ChIP-qPCR). Yeast-one-hybrid experiments revealed that the ERF2-like protein could bind to the *NaEAS12* promoter fragment *EAS12-b* (located from -699 to -926 bp upstream of the starting codon), which

1 contained the ATCTA motif (Fig. 9). Further EMSA experiments indicated that
 2 NaERF2-like could directly bind to the biotin labeled probe EM13 (5'-
 3 -tagattATCTAattctact-3'), but not to the mutated one (5'-tagattAATTAattctact-3')(Fig.
 4 9). To further confirm these results *in vivo*, we generated a transgenic line
 5 ectopically-expressing the NaERF2-like protein fused with two HA tags at the
 6 C-terminal (35S:: NaERF2-like-2HA) for use in ChIP-qPCR. The fusion protein could
 7 be detected by a commercial HA antibody (Supplemental Fig. S2), suggesting that the
 8 protein was successfully over-expressed in *N. attenuata* plants. Transgenic plants
 9 were inoculated with *A. alternata* and sampled at 1 dpi. We found that the HA-tagged
 10 NaERF2-like protein bound the *NaEAS12* promoter at a site which encompassed the
 11 ACTCA motif (Fig. 9).

12

13 **Over-expression of *NaERF2-like* does not alter plant resistance, but increases *A.*** 14 ***alternata*-induced *NaEAS12* gene expression and capsidiol levels**

15 To further understand the role of *NaERF2-like* in pathogen defense we
 16 investigated *NaEAS12* gene expression, capsidiol level and plant resistance in WT,
 17 and two *NaERF2-like* over-expression (Ov-NaERF2-like) lines. The ectopic
 18 over-expression of *NaERF2-like* fused with two HA tags substantially increased the
 19 basal and induced transcriptional levels of *NaERF2-like* (Fig. 10A) without affecting
 20 the plant's morphology and size. As expected, Ov-NaERF2-like line 1 and
 21 Ov-NaERF2-like line 2 showed 178% and 219% of the *NaEAS12* expression of WT
 22 when 0 leaves were inoculated at 1 d (Fig. 10A). Consistently, capsidiol levels
 23 attained values 149% and 175% of that of WT in Ov-NaERF2-like lines 1 and 2 at 3
 24 dpi, respectively (Fig. 10B). However, we found no difference in lesion diameters in
 25 both over-expression lines compared with WT (Supplementary Fig. S2), indicating
 26 that over-expression of *NaERF2-like* increased the gene expression of *NaEAS12*, and
 27 subsequently capsidiol biosynthesis, but had only a minor effect on the plants'
 28 resistance.

29

Discussion

Phytoalexins are important ‘chemical weapons’ employed by plants in defending against pathogens. In addition to scopoletin and scopolin, two phytoalexins regulated by JA and ethylene signaling pathways in response to *A. alternata* infection (Sun *et al.*, 2014b; Li and Wu, 2016), we demonstrate in this study that capsidiol is another important phytoalexin produced by *N. attenuata*, and its biosynthesis is not dependent on JA and ethylene signaling pathways but is transcriptionally regulated by a transcription factor NaERF2-like.

Capsidiol is an important phytoalexin produced in *N. attenuata* in response to *A. alternata* infection

Capsidiol was initially isolated from pepper fruit after treatments of various pathogens, including *Phytophthora capsici*, *Botrytis cinerea*, and *Fusarium oxysporum* (Stoessl *et al.*, 1972). Later, this compound was also identified from infected *Nicotiana* species (Bailey *et al.*, 1975; Guedes *et al.*, 1982; Mialoundama *et al.*, 2009; Shibata *et al.*, 2010; Grosskinsky *et al.*, 2011; Shibata *et al.*, 2016). In our *N. attenuata* -*A. alternata* pathosystem, we found that a large number of genes were strongly regulated in response to *A. alternata* inoculation at 1 dpi during transcriptome analysis. Many of these genes were involved in the biosynthesis of sesquiterpenes, capsidiol and solavetivone (Fig. 1 and Supplementary Table S1). Indeed, the levels of capsidiol were dramatically induced to $50.68 \pm 3.10 \mu\text{g/g}$ fresh leaves at 3 dpi in young 0 leaves (Fig. 2). These finding suggest that capsidiol is involved in the resistance of *N. attenuata* to *A. alternata* infection.

Ideally, the benefits of a putative resistant trait should be determined in plants differing only in a single gene that controls the defense trait and are otherwise identical (Bergelson *et al.*, 1996). In this study, virus-induced gene silencing of NaEAHs or NaEASs was used to manipulate the production of capsidiol, and the results revealed that capsidiol-reduced or -depleted plants were more susceptible to *A. alternata* (Fig. 3). In addition, capsidiol showed strong anti-fungal activities against *A. alternata* when it was extracted and purified from infected plants and applied to

1 fungal growth *in vitro* at a concentration in the range of observed *in planta* (Fig. 4).

2 Thus, our results demonstrate that capsidiol is an important phytoalexin involved
3 in the defense mechanism of *N. attenuata* against *A. alternata*, and the high level of
4 capsidiol accumulated in the young 0 leaves accounts for their high resistance to *A.*
5 *alternata* infection. Whether solavetivone plays a role in resistance is unknown.
6 Transcriptome data, especially the strong up-regulation of *premnaspirodiene*
7 *oxygenases* and *premnaspirodiene synthases* at 1 dpi (Fig. 1 and Supplementary Table
8 S1), is consistent with a defensive role of solavetivone. However, this question needs
9 further investigation.

11 Regulation of capsidiol biosynthesis

12 Several reports indicate that the phytoalexin production is influenced by
13 endogenous plant hormones. Increasing cytokinins (CK) levels in *N. tabacum* plants
14 by exogenous CK application or overexpression of bacterial *isopentenyl transferase*
15 gene enhanced their resistance to the hemibiotrophic bacterium, *Pseudomonas syringae*,
16 by increasing levels of capsidiol and scopoletin (Grosskinsky *et al.*, 2011). This
17 CK-mediated resistance is independent of salicylic acid, jasmonate and ethylene
18 signaling pathways (Grosskinsky *et al.*, 2011). Absciscic acid (ABA) negatively
19 regulates elicitor-induced biosynthesis of capsidiol in *N. plumbaginifolia*; a two-fold
20 increase in capsidiol synthesis was observed in ABA-deficient mutants compared with
21 WT plants when exposed to cellulose or *B. cinerea* (Mialoundama *et al.*, 2009). In
22 addition, when *N. benthamiana* was inoculated with *Phytophthora infestans*,
23 pathogen-induced capsidiol and *NbEAS* and *NbEAH* expression were abolished in
24 plants silenced with *ethylene insensitive 2* (*NbEIN2*), suggesting that ethylene
25 signaling pathway is essential for capsidiol production (Shibata *et al.*, 2010; Ohtsu *et*
26 *al.*, 2014).

27 In contrast to the observation in *N. benthamiana* inoculated with *P. infestans*, our
28 experiments did not support the role of ethylene signaling in capsidiol elicitation in *N.*
29 *attenuata*-*A. alternata* pathosystem. When *NaEIN2* was silenced by VIGS, *A.*
30 *alternata*-elicited *NaF6'H1* was dramatically reduced, which is consistent with our

1 previous finding that ethylene signaling is essential for scopoletin biosynthesis (Sun *et*
2 *al.*, 2017), but *NaEAHs* and *NaEASs* transcripts were induced to levels similar to plants
3 transformed with empty vector (Supplementary Fig. S3). This result indicates that
4 blocking the ethylene signaling pathway has little effect on the expression of these
5 two key capsidiol biosynthesis genes. Additional evidence against the involvement of
6 ethylene signaling comes from *irACO* plants (ethylene-deficient) and *Ov-etr1* plants
7 (ethylene-insensitive), both of which were generated previous by von Dahl *et al*
8 (2007). Both capsidiol production and transcripts of *NaEAHs* and *NaEASs* were
9 induced to the same high levels in WT, *irACO* and *Ov-etr1* plants (Fig. 5). Thus, we
10 concluded that ethylene signaling pathway does not play a critical role in capsidiol
11 production and gene expression of *NaEAHs* and *NaEASs* in *N. attenuata* after *A.*
12 *alternata* challenge. Whether or not ethylene is involved in capsidiol biosynthesis is
13 likely dependent on pathosystem. Additionally, our experiments do not indicate a role
14 for jasmonate signaling in the regulation of capsidiol biosynthesis, as transcripts of
15 *NaEAHs* and *NaEASs* was equivalent in WT and JA deficient *irAOC* plants at 1 and 3
16 dpi (Fig. 5).

17 Despite the great role of capsidiol on resistance, the transcriptional regulation of
18 its biosynthesis is still not clear. Due to the high level of gene expression seen in the
19 capsidiol biosynthetic pathway, we hypothesized that the transcription factors
20 regulating these genes must also be highly expressed during the initial infection
21 period of the fungus. We performed a screen of the 6 most up-regulated transcription
22 factors. Indeed, the *NaERF2-like* was identified to be a positive regulator of plant
23 resistance and capsidiol production. When compared with EV plants, those with a
24 silenced *NaERF2-like* gene accumulated fewer transcripts of *NaEASs* and *NaEAHs*, as
25 well as lower levels of capsidiol (Fig. 7). Consistently, *NaERF2-like*-silenced plants
26 were susceptible to the fungus (Fig. 7).

27 Both *NaEAS* and *NaEAH* are encoded by members of a multi-gene family. Since
28 *NaEAS12* expression is greatly reduced in *NaERF2-like*-silenced plants (Fig. 8), we
29 selected this gene to test whether its promoter could be directly activated by
30 *NaERF2-like*. Several lines of evidence support that *NaERF2-like* directly regulates

the capsidiol biosynthetic gene *NaEAS12*, including 1) the binding of NaERF2-like protein to the promoter region of *NaEAS12*, which was supported by yeast-one-hybrid, EMSA and Chip-qPCR experiments (Fig. 9); 2) the *NaEAS12* promoter was activated in response to transient *NaERF2-like* over-expression (Fig. 8); this result is further confirmed by stable transgenic lines of Ov-*NaERF2-like*, which exhibit increased *NaEAS12* transcripts and higher levels of capsidiol accumulation (Fig. 10). Currently, it is not known how other *NaEAS*s and *NaEAH*s are regulated by NaERF2-like. Further experiments are needed to test whether or not they are regulated in a way similar to *NaEAS12*.

Accession Numbers

Sequence data from this article can be found in the GeneBank data library under accession numbers: XM_019375732.1 (HMG-CoA synthase), XM_019375278.1 (MVAPP decarboxylase), XM_019403732.1 (FPP synthase), XM_019409657.1 (Squalene synthase), XM_019408556.1 (*EAS12*), XM_019399671.1 (NaERF2-like).

Supplementary data:

Fig. S1. *Alternaria alternata*-elicited *NaEAS12* transcripts in EV and plants individually silenced with the top 5 up-regulated transcription factors.

Mean ($\pm$ SE) relative *A. alternata*-induced *NaEAS12* transcripts as measured by real-time PCR in 5 replicated young leaves of EV, VIGS NaERF ABR1-like, VIGS NaZAT12-like, VIGS NaWRKY40, VIGS NaWRKY43 and VIGS NaWRKY61 plants at 3 dpi. Two independent VIGS experiments presented similar results. Asterisks indicate levels of significant differences between EV and VIGS plants (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$)

Fig. S2. Overexpression of NaERF2-like does not affect plant resistance.

NaERF2-like proteins were detected in 0 leaves of Ov-*NaERF2-like* line 1 and 2 at 1

dpi by HA antibody via western blot (A). Mean ($\pm$ SE) diameter of necrotic lesions (B) was recorded in 8-replicated 0 leaves of WT, Ov-NaERF2-like line 1 and 2 plants inoculated with *A. alternata* for 5 d.

Fig. S3. Silencing *NaEIN2* has a great impact on *A. alternata*-induced transcripts of *NaF6'H1* but does not affect transcripts of *NaEAHs* and *NaEASs*.

Mean ($\pm$ SE) relative *A. alternata*-induced *NaEIN2*, *NaF6'H1*, *NaEASs*, *NaEAHs* transcripts as measured by real-time PCR in 5 replicated young leaves of EV and VIGS *NaEIN2* plants at 3 dpi. Asterisks indicate levels of significant differences between EV and VIGS plants with the same treatments (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$)

Table S1.

Transcriptome analysis revealed regulation of genes involved in sesquiterpene biosynthesis in 3 biological replicate *N. attenuata* leaves after *A. alternata* inoculation at 1 d.

Table S2.

Transcriptome analysis revealed top 6 highly elicited transcriptional factor genes in 3 biological replicate *A. alternata*-inoculated *N. attenuata* leaves at 1 d.

Table S3.

Primers used in this study.

Acknowledgements: We thank Prof. Joe Chappell (University of Kentucky, USA) for the capsidiol standard, the Service Center for Experimental Biotechnology of Kunming Institute of Botany, the Chinese Academy of Sciences (CAS), for plant growth support. This project was supported by the National Science Foundation of China (NSFC Grant No. 31670262) and the Key Project of Applied Basic Research Program of Yunnan (2014FA040) to Prof. Jinsong Wu, NSFC grant (No. 31700231),

1 the Applied Basic Research Program of Yunnan (2017FB048) and CAS "Light of
2 West China" Program to Dr. Lan Ma.

3 **References**

- 4 **Ahuja I, Kissen R, Bones AM.** 2012. Phytoalexins in defense against pathogens.
5 *Trends in Plant Science*, **17**, 73-90.
- 6 **Bailey JA, Burden RS, Vincent GG.** 1975. Capsidiol: Antifungal compound
7 produced in *Nicotiana tabacum* and *Nicotiana clevelandii* following infection
8 with tobacco necrosis virus. *Phytochemistry*, **14**, 597-597.
- 9 **Bergelson J, Purrington CB, Palm CJ, Lopez-Gutierrez JC.** 1996. Costs of
10 resistance: a test using transgenic *Arabidopsis thaliana*. *Proc Biol Sci*, **263**,
11 1659-1663.
- 12 **Berrocal-Lobo M, Molina A, Solano R.** 2002. Constitutive expression of
13 ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to
14 several necrotrophic fungi. *The Plant Journal*, **29**, 23-32.
- 15 **El Oirdi M, Trapani A, Bouarab K.** 2010. The nature of tobacco resistance against
16 *Botrytis cinerea* depends on the infection structures of the pathogen.
17 *Environmental Microbiology*, **12**, 239-253.
- 18 **Facchini PJ, Chappell J.** 1992. Gene family for an elicitor-induced sesquiterpene
19 cyclase in tobacco. *Proceedings of the National Academy of Sciences of the*
20 *United States of America*, **89**, 11088-11092.
- 21 **Glazebrook J.** 2005. Contrasting mechanisms of defense against biotrophic and
22 necrotrophic pathogens. *Annual Review Phytopathology*, **43**, 205-227.
- 23 **Grosskinsky DK, Naseem M, Abdelmohsen UR, Plickert N, Engelke T, Griebel T,**
24 **Zeier J, Novak O, Strnad M, Pfeifhofer H, van der Graaff E, Simon U,**
25 **Roitsch T.** 2011. Cytokinins mediate resistance against *Pseudomonas syringae*
26 in tobacco through increased antimicrobial phytoalexin synthesis independent
27 of salicylic acid signaling. *Plant Physiology*, **157**, 815-830.
- 28 **Guedes MEM, Kuc J, Hammerschmidt R, Bostock R.** 1982. Accumulation of 6
29 sesquiterpenoid phytoalexins in tobacco leaves infiltrated with *Pseudomonas*

- 1 lachrymans. *Phytochemistry*, **21**, 2987-2988.
- 2 **Hao D, Ohme-Takagi M, Sarai A.** 1998. Unique mode of GCC box recognition by
- 3 the DNA-binding domain of ethylene-responsive element-binding factor (ERF
- 4 domain) in plant. *Journal of Biological Chemistry*, **273**, 26857-26861.
- 5 **Hao D, Yamasaki K, Sarai A, Ohme-Takagi M.** 2002. Determinants in the sequence
- 6 specific binding of two plant transcription factors, CBF1 and NtERF2, to the
- 7 DRE and GCC motifs. *Biochemistry*, **41**, 4202-4208.
- 8 **Huang PY, Catinot J, Zimmerli L.** 2016. Ethylene response factors in Arabidopsis
- 9 immunity. *Journal of Experimental Botany*, **67**, 1231-1241.
- 10 **Kallenbach M, Bonaventure G, Gilardoni PA, Wissgott A, Baldwin IT.** 2012.
- 11 Empoasca leafhoppers attack wild tobacco plants in a jasmonate-dependent
- 12 manner and identify jasmonate mutants in natural populations. *Proceedings of*
- 13 *the National Academy of Sciences*, **109**, E1548-57.
- 14 **Kliebenstein DJ, Rowe HC, Denby KJ.** 2005. Secondary metabolites influence
- 15 Arabidopsis/Botrytis interactions: variation in host production and pathogen
- 16 sensitivity. *The Plant Journal*, **44**, 12.
- 17 **Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT.** 2002.
- 18 Agrobacterium-mediated transformation of *Nicotiana attenuata*, a model
- 19 ecological expression system. *Chemoecology*, **12**, 177-183
- 20 **LaMondia JA.** 2001. Outbreak of brown spot of tobacco caused by *Alternaria*
- 21 *alternata* in Connecticut and Massachusetts. *Plant Disease*, **85**, 230-230.
- 22 **Li J, Wu J.** 2016. Scopolin, a glycoside form of the phytoalexin scopoletin, is likely
- 23 involved in the resistance of *Nicotiana attenuata* against *Alternaria alternata*.
- 24 *Journal of Plant Pathology*, **98**, 641-644.
- 25 **Mengiste T.** 2012. Plant immunity to necrotrophs. *Annual Review of Phytopathology*,
- 26 **50**, 267-294.
- 27 **Mialoundama AS, Heintz D, Debayle D, Rahier A, Camara B, Bouvier F.** 2009.
- 28 Absciscic acid negatively regulates elicitor-induced synthesis of capsidiol in
- 29 wild tobacco. *Plant Physiology*, **150**, 1556-1566.
- 30 **Nafisi M, Goregaoker S, Botanga CJ, Glawischnig E, Olsen CE, Halkier BA,**

- 1 **Glazebrook J.** 2007. Arabidopsis cytochrome P450 monooxygenase 71A13
2 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis.
3 *Plant Cell*, **19**, 2039-2052.
- 4 **Ohtsu M, Shibata Y, Ojika M, Tamura K, Hara-Nishimura I, Mori H, Kawakita**
5 **K, Takemoto D.** 2014. Nucleoporin 75 is involved in the ethylene-mediated
6 production of phytoalexin for the resistance of *Nicotiana benthamiana* to
7 *Phytophthora infestans*. *Molecular Plant-Microbe Interactions*, **27**, 1318-1330.
- 8 **Pre M, Atallah M, Champion A, De Vos M, Pieterse CMJ, Memelink J.** 2008. The
9 AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and
10 ethylene signals in plant defense. *Plant Physiology*, **147**, 1347-1357.
- 11 **Ralston L, Kwon ST, Schoenbeck M, Ralston J, Schenk DJ, Coates RM,**
12 **Chappell J.** 2001. Cloning, heterologous expression, and functional
13 characterization of 5-epi-aristolochene-1,3-dihydroxylase from tobacco
14 (*Nicotiana tabacum*). *Archives of Biochemistry and Biophysics*, **393**, 222-235.
- 15 **Sanchez-Vallet A, Ramos B, Bednarek P, Lopez G, Pislewska-Bednarek M,**
16 **Schulze-Lefert P, Molina A.** 2010. Tryptophan-derived secondary metabolites
17 in *Arabidopsis thaliana* confer non-host resistance to necrotrophic
18 *Plectosphaerella cucumerina* fungi. *The Plant Journal*, **63**, 115-127.
- 19 **Schuck S, Weinhold A, Luu VT, Baldwin IT.** 2014. Isolating fungal pathogens from
20 a dynamic disease outbreak in a native plant population to establish
21 plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*.
22 *PLoS One*, **9**, e102915.
- 23 **Shibata Y, Kawakita K, Takemoto D.** 2010. Age-related resistance of *Nicotiana*
24 *benthamiana* against hemibiotrophic pathogen *Phytophthora infestans* requires
25 both ethylene- and salicylic acid-mediated signaling pathways. *Molecular*
26 *Plant-Microbe Interactions*, **23**, 1130-1142.
- 27 **Shibata Y, Ojika M, Sugiyama A, Yazaki K, Jones DA, Kawakita K, Takemoto D.**
28 2016. The full-size ABCG transporters Nb-ABCG1 and Nb-ABCG2 function
29 in pre- and post-invasion defense against *Phytophthora infestans* in *Nicotiana*
30 *benthamiana*. *Plant Cell*, **28**, 1163–1181.

- 1 **Solano R, Stepanova A, Chao Q, Ecker JR.** 1998. Nuclear events in ethylene
2 signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3
3 and ETHYLENE-RESPONSE-FACTOR1. *Genes & Development*, **12**,
4 3703-3714.
- 5 **Stoessl A, Unwin CH, Ward EWB.** 1972. Postinfectious inhibitors from plants I.
6 Capsidiol, an antifungal compound from capsicum frutescens. *Phytopathology*,
7 **74**, 141-152.
- 8 **Sun H, Hu X, Ma J, Hettenhausen C, Wang L, Sun G, Wu J, Wu J.** 2014a.
9 Requirement of ABA signalling-mediated stomatal closure for resistance of
10 wild tobacco to *Alternaria alternata*. *Plant Pathology*, **63**, 1070-1077.
- 11 **Sun H, Wang L, Zhang B, Ma J, Hettenhausen C, Cao G, Sun G, Wu J, Wu J.**
12 2014b. Scopoletin is a phytoalexin against *Alternaria alternata* in wild tobacco
13 dependent on jasmonate signalling. *Journal of Experimental Botany*, **65**,
14 4305-4315.
- 15 **Sun H, Song N, Ma L, Li J, Ma L, Wu J, Wu J.** 2017. Ethylene signalling is
16 essential for the resistance of *Nicotiana attenuata* against *Alternaria alternata*
17 and phytoalexin scopoletin biosynthesis. *Plant Pathology*, **66**, 277-284.
- 18 **von Dahl CC, Winz RA, Halitschke R, Kuhnemann F, Gase K, Baldwin IT.** 2007.
19 Tuning the herbivore-induced ethylene burst: the role of transcript
20 accumulation and ethylene perception in *Nicotiana attenuata*. *The Plant*
21 *Journal*, **51**, 293-307.
- 22 **Ward EWB, Unwin CH, Stoessl A.** 1974. Postinfectious inhibitors from plants 13.
23 Fungitoxicity of phytoalexin, capsidiol, and related sesquiterpenes. *Canadian*
24 *Journal of Botany-Revue Canadienne De Botanique*, **52**, 2481-2488.
- 25 **Welsch R, Maass D, Voegel T, Dellapenna D, Beyer P.** 2007. Transcription factor
26 RAP2.2 and its interacting partner SINAT2: stable elements in the
27 carotenogenesis of *Arabidopsis* leaves. *Plant Physiology*, **145**, 1073-1085.
- 28 **Wu J, Wang L, Wunsche H, Baldwin IT.** 2013. Narboh D, a respiratory burst
29 oxidase homolog in *Nicotiana attenuata*, is required for late defense responses
30 after herbivore attack. *Journal of Integrative Plant Biology*, **55**, 187-198.

- 1 **Xu Z, Song N, Ma L, Fang D, Wu J.** 2018. NaPDR1 and NaPDR1-like are essential
2 for *Nicotiana attenuata* resistance to the fungal pathogen *Alternaria alternata*.
3 *Plant Diversity*, **40**, 68-73.
- 4 **Yoo SD, Cho YH, Sheen J.** 2007. Arabidopsis mesophyll protoplasts: a versatile cell
5 system for transient gene expression analysis. *Nature Protocols*, **2**, 1565-1572.
- 6 **Zhao Y, Wei T, Yin KQ, Chen Z, Gu H, Qu LJ, Qin G.** 2012. Arabidopsis RAP2.2
7 plays an important role in plant resistance to *Botrytis cinerea* and ethylene
8 responses. *New Phytologist*, **195**, 450-460.

9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

Figure legends

Fig. 1 The regulation of terpene biosynthetic genes in *N. attenuata* in response to *A. alternata* inoculation at 1 day post inoculation (dpi)

Genes involved in terpene synthesis were strongly regulated during transcriptome analysis in 3 biological replicate samples of mock and 1 dpi: transcripts of all enzymes marked in red font were up-regulated at 1 dpi, while *squalene synthase* (*SS*) with blue font was down-regulated (A). To validate this regulation, relative mean transcripts ($\pm$ SE) of *HMG-CoA reductase* (A), *MVAPP decarboxylase* (B), *FPPS* (C), *squalene synthase* (D), *EASs* (E) and *EAHs* (F) were measured by real-time PCR in 4 biological replicate 0 leaves at 1 dpi. Leaves without inoculation were collected as controls (Mock 1 d). Both *EASs* and *EAHs* were detected by primers conserved in gene family members. Asterisks indicate the level of significant differences between Mock 1 d and 1 dpi samples (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$). Enzymes/substrates: 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), 3-hydroxy-3-methyl-glutaryl-CoA synthase (HMG-CoA synthase), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), mevalonic acid kinase (MVA kinase), mevalonic acid 5-phosphate kinase (MVAP kinase), mevalonic acid 5-diphosphate decarboxylase (MVAPP decarboxylase), isopentenyl diphosphate (IPP), 3'-dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl pyrophosphate synthase (FPPS), farnesyl diphosphate (FPP), 5-*epi*-aristolochene (EA), 5-*epi*-aristolochene synthase (EAS), 5-*epi*-aristolochene hydroxylase (EAH), prenaspirodiene synthase (PS), prenaspirodiene oxidase (PO), geranylgeranyl diphosphate (GGPP).

Fig. 2 Accumulation of capsidiol in 0 (young) and +3 (mature) leaves after *A. alternata* inoculation

Mean ($\pm$ SE) capsidiol levels were determined by HPLC in 5 biological replicate 0 and +3 leaves at 1 and 3 dpi. Asterisks indicate levels of significant differences

1 between mock and infected samples (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***,
2 $p < 0.005$). N.D., not detectable.

3

4 **Fig. 3 Silencing *NaEAHs* or *NaEASs* expressions dramatically reduces *A.***
5 ***alternata*-induced capsidiol levels and plant resistance without affecting**
6 **scopoletin.**

7 (A): Mean ($\pm$ SE) relative *A. alternata*-induced *NaEAHs* and *NaEASs* transcripts as
8 measured by real-time PCR in 5 replicate young leaves of EV, VIGS *NaEAHs* and
9 VIGS *NaEASs* plants at 2 dpi.

10 (B): Mean ($\pm$ SE) capsidiol and scopoletin (including scopolin) levels were
11 determined by HPLC in 5 replicate young leaves of EV, VIGS *NaEAHs* and VIGS
12 *NaEASs* plants at at 3 dpi.

13 (C): Mean ($\pm$ SE) diameter of necrotic lesions in 8 replicate young leaves of EV,
14 VIGS *NaEAHs* and VIGS *NaEASs* plants infected with *A. alternata* for 6 d.

15 As both *NaEAH* and *NaEAS* are encoded by large gene families, conserved cDNA
16 regions were used for silencing and real time PCR. The asterisks indicate levels of
17 significant differences between EV and VIGS leaves (Student's *t*-test: *, $p < 0.05$; ***,
18 $p < 0.005$). N.D., not detectable.

19

20 **Fig. 4 Capsidiol exhibits anti-fungal activity *in vitro*.**

21 (A): Growth of *A. alternata* mycelium at day 6 in PDA with capsidiol at final
22 concentration of 0, 50, 100, and 200 $\mu\text{g/mL}$. PDA plates with 1 % of methanol were
23 served as controls.

24 (B): The area of *A. alternata* mycelium growth in PDA with different concentrations
25 of capsidiol was determined by ImageJ. Data were collected every 2 d.
26 Asterisks indicate levels of significant differences between mock and infected
27 samples (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

28

29 **Fig. 5 JA and ethylene pathways play a minor role in *A. alternata*-induced**
30 **transcripts of *NaEASs* and *NaEAHs*, and capsidiol biosynthesis.**

1 Mean ($\pm$ SE) relative *A. alternata*-induced *NaEASs* (A) and *NaEAHs* (B) transcripts
2 as measured by real-time PCR in 5 biological replicates of young leaves (0 leaves) in
3 WT, irAOC (JA-deficient), irACO (ethylene deficient) and Ov-etr1
4 (ethylene-insensitive) plants at 1 and 3 dpi. Mean ($\pm$ SE) capsidiol levels (C) were
5 determined by HPLC in 5 replicated 0 leaves of WT, irAOC, irACO and Ov-etr1
6 plants at 3 dpi. N.D., not detectable.

7
8 **Fig. 6 NaERF2-like is highly elicited after *A. alternata* inoculation and is targeted**
9 **to the nucleus.**

10 (A): Mean ($\pm$ SE) relative *A. alternata*-induced *NaERF2-like* transcripts as measured
11 by real-time PCR in 4 replicate 0 leaves at 1 and 3 dpi. The asterisks indicate levels of
12 significant differences between mock and infected samples (Student's *t*-test: *, $p < 0.05$;
13 ***, $p < 0.005$).

14 (B): Two fusion proteins, 35S::eGFP and 35S::NaERF2-like-eGFP, were expressed
15 transiently in *N. attenuata* protoplasts, respectively. NaERF2-like was found to be
16 targeted to the nucleus. Images were taken in bright field (left), UV (middle) and GFP
17 channels (right).

18
19 **Fig. 7 Silencing *NaERF2-like* impairs *A. alternata*-induced transcripts of *NaEASs***
20 **and *NaEAHs*, capsidiol levels and plant resistance without affecting *NaF6'H1***
21 **transcript accumulation.**

22 Mean ($\pm$ SE) relative *A. alternata*-induced *NaERF2-like* (A), *NaEASs* (B), *NaEAHs*
23 (C) and *NaF6'H1* (D) transcript abundance as measured by real-time PCR in 5
24 replicate young leaves of EV and VIGS *NaERF2-like* plants at 2 dpi; capsidiol levels
25 (E) were determined by HPLC in 5 replicate young leaves of EV and VIGS
26 *NaERF2-like* plants at 3 dpi; mean ($\pm$ SE) diameter of necrotic lesions (F) were
27 recorded in 8 replicate young leaves of EV and VIGS *NaERF2-like* plants infected
28 with *A. alternata* for 6 d. Two independent VIGS experiments returned similar results.
29 The asterisks indicate levels of significant differences between EV and VIGS leaves

1 (Student's *t*-test: *, $p < 0.05$; ***, $p < 0.005$). N.D., not detectable.

2

3 **Fig. 8 A. *alternata*-elicited *NaEAS12* transcripts are largely dependent on**
 4 ***NaERF2-like*, and transient over-expression of *NaERF2-like* leads to**
 5 **transactivation of *NaEAS12* promoter**

6 (A): Mean ($\pm$ SE) relative *A. alternata*-induced *NaEAS12* transcripts as measured by
 7 real-time PCR in 5 replicate young leaves of EV and VIGS *NaERF2-like* plants at 2
 8 dpi. The asterisks indicate levels of significant differences between EV and VIGS
 9 leaves at 2 dpi (Student's *t*-test: ***, $p < 0.005$).

10 (B): Transcript abundance of *NaERF2-like*-2HA-eGFP and *LUC* in *N. attenuata*
 11 protoplasts co-expressing *NaEAS12* promoter::LUC and 35S::2HA-eGFP or 35S::
 12 *NaERF2-like*-2HA-eGFP, were measured in 3 biological samples. Experiments were
 13 repeated twice with similar results. The asterisks indicate levels of significant
 14 differences between control and *NaERF2-like* over-expressing protoplasts (Student's
 15 *t*-test: *, $p < 0.05$; ***, $p < 0.005$).

16

17 **Fig. 9 The binding of *NaERF2-like* and *NaEAS12* promoter as demonstrated by**
 18 **yeast-one-hybrid, EMSA and chromatin immunoprecipitation**

19 The *NaEAS12* promoter structure indicated the pEAS12-b (-669 to -926 numbered
 20 from the ATG) for yeast-one hybrid, the probe EM13 (5'-tagattATCTaattctact-3') for
 21 EMSA, and the pEAS12-13 (-669 to -781 numbered from the ATG) for ChIP assays.
 22 Yeast-one-hybrid analysis revealed that *NaERF2-like* could bind to EAS12-b as the
 23 yeast cells could grow on the SD/-His/-Leu medium supplied with 200 ng/mL (final
 24 conc.) 3-AT; Y1HGold [pGADT7/ pEAS12-b-AbAi] was used as a negative control,
 25 and Y1HGold [pGADT7 Rec-p53/p53-AbAi] was used as a positive control.
 26 EMSA demonstrated that His tagged *NaERF2-like* could bind to probe EM13 but not
 27 to the mutated one. The mutant probe (5'-tagattAATTaattctact-3') served as a negative
 28 control in EMSA.

29 ChIP-real time PCR data indicated *NaERF2-like* bound to the promoter of *NaEAS12*.

30 Negative controls were without antibody (No Ab) and with HA antibody but using

primers detecting *NaActin 2*. The asterisks indicate levels of significant differences between No Ab and with Ab in pEAS12-13 (Student's *t*-test: **, $p < 0.01$).

Fig. 10 Over-expression of *NaERF2-like* enhances *NaEAS12* expression and capsidiol production in stable transformation plants.

(A): Mean ($\pm$ SE) relative *A. alternata*-induced *NaERF2-like* and *NaEAS12* transcripts as measured by real-time PCR in 5 replicate 0 leaves of WT, Ov-*NaERF2-like* line 1 and Ov-*NaERF2-like* line 2 plants at 1 dpi.

(B): Mean ($\pm$ SE) capsidiol levels were determined by HPLC in 5 replicate 0 leaves of WT, Ov-*NaERF2-like* line 1 and Ov-*NaERF2-like* line 2 plants at 3 dpi.

The asterisks indicate levels of significant differences between WT and Ov-*NaERF2-like* lines after inoculation (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$). N.D., not detectable.

Figures

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

1

2

3

4 **Fig. 10.**

5