

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

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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.

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1 **Abstract**

2 Capsidiol is a sesquiterpenoid phytoalexin produced in *Nicotiana* and *Capsicum*
3 species in response to pathogen attack. Whether capsidiol plays a defensive role and
4 how its biosynthesis is regulated in the wild tobacco *Nicotiana attenuata* when the
5 plant is attacked by *Alternaria alternata* (tobacco pathotype), a notorious necrotrophic
6 fungus causing brown spot disease, is unknown. Transcriptome analysis indicated that
7 a metabolic switch to sesquiterpene biosynthesis occurred in young leaves of *N.*
8 *attenuata* after *A. alternata* inoculation: many genes leading to sesquiterpene
9 production were strongly up-regulated, including the capsidiol biosynthetic genes,
10 *5-epi-aristolochene synthase (EAS)* and *5-epi-aristolochene hydroxylase (EAH)*.
11 Consistently, the level of capsidiol was increased dramatically in young leaves after
12 fungal inoculation, from not detectable in mock control to $50.68 \pm 3.10 \mu\text{g/g}$ fresh
13 leaves at 3 days post inoculation. Capsidiol-reduced or capsidiol-depleted plants,
14 which were generated by silencing *EAHs* or *EASs* by virus-induced gene silencing,
15 were more susceptible to the fungus. In addition, this sesquiterpene exhibited strong
16 anti-fungal activities against *A. alternata in vitro* when purified from infected plants
17 and applied to fungal growth. Furthermore, an ERF2-like transcription factor was
18 found to positively regulate capsidiol production and plant resistance through the
19 direct transactivation of a capsidiol biosynthetic gene *EAS12*. Taken together, our
20 results demonstrate that capsidiol, a phytoalexin highly accumulated in *N. attenuata*
21 plants in response to *A. alternata* infection, plays an important role in pathogen
22 resistance independent of JA and ethylene signaling pathways, and its biosynthesis is
23 transcriptionally regulated by an ERF2-like transcription factor.

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

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1 **Introduction**

2 Plants are constantly attacked by a wide variety of microbial pathogens. In
3 response, they activate a large number of intricate defense mechanisms, including the
4 formation of reactive oxygen species, physical reinforcement of cell walls, production
5 of phytohormones, antimicrobial proteins and metabolites (Glazebrook, 2005; Ahuja
6 *et al.*, 2012; Mengiste, 2012). The class of small molecules known as phytoalexins is
7 produced by plants *de novo* in response to pathogen attack, and is an important part of
8 the plant defense repertoire (Ahuja *et al.*, 2012). In *Arabidopsis*, mutants impaired in
9 the production of the phytoalexin camalexin are more susceptible to infection by
10 necrotrophic fungi, such as *Alternaria brassicicola* (Nafisi *et al.*, 2007), *Botrytis*
11 *cinerea* (Kliebenstein *et al.*, 2005) and *Plectosphaerella cucumerina* (Sanchez-Vallet *et*
12 *al.*, 2010). In *Nicotiana* species, two phytoalexins have recently received attention:
13 scopoletin (El Oirdi *et al.*, 2010; Sun *et al.*, 2014b) and capsidiol (Mialoundama *et al.*,
14 2009; Shibata *et al.*, 2010; Grosskinsky *et al.*, 2011; Shibata *et al.*, 2016).

15 Capsidiol has been proposed to be an important ‘chemical weapon’ against
16 pathogens in *Nicotiana* species. This bicyclic sesquiterpene is produced via
17 cyclization of farnesyl pyrophosphate (FPP) to 5-*epi*-aristolochene by
18 5-*epi*-aristolochene synthase (EAS), followed by two hydroxylation reactions
19 catalyzed by 5-*epi*-aristolochene dihydroxylase (EAH) (Facchini and Chappell, 1992;
20 Ralston *et al.*, 2001). Capsidiol exhibits toxicity towards many pathogens, including
21 *Phytophthora capsici* and *B. cinerea* (Stoessl *et al.*, 1972; Ward *et al.*, 1974). Recently,
22 molecular evidence also supports its role in non-host resistance against *P. infestans* in
23 *N. benthamiana*, as *NbEAS*- or *NbEAH*-silenced plants were highly susceptible
24 (Shibata *et al.*, 2010).

25 Ethylene response factors (ERFs) are transcription factors that play crucial roles
26 in plant immunity (Huang *et al.*, 2016). In *Arabidopsis*, several ERFs have been
27 identified as important regulators in *Botrytis* resistance, such as ORA59, ERF1 and
28 RAP2.2 (Solano *et al.*, 1998; Berrocal-Lobo *et al.*, 2002; Pre *et al.*, 2008; Zhao *et al.*,
29 2012). Most ERFs are able to bind specifically to DNA sequences containing a GCC
30 (GCC box) and/or a dehydration-responsive element/C-repeat (DRE/CRT box,

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

4 The necrotrophic fungal pathogen *Alternaria alternata* (tobacco pathotype)
5 causes brown spot disease in *Nicotiana tabacum* (LaMondia, 2001) and many other
6 *Nicotiana* species, including the wild tobacco *Nicotiana attenuata* (Schuck *et al.*,
7 2014). Inoculation with *A. alternata* elicits the activation of both jasmonate and
8 ethylene signaling pathways in *N. attenuata* plants, which subsequently lead to the
9 accumulation of the phytoalexins, scopoletin and its glycoside form, scopolin (Sun *et*
10 *al.*, 2014b; Li and Wu, 2016; Sun *et al.*, 2017). Currently it is not known if
11 fungus-inoculated *N. attenuata* plants produce other phytoalexins, such as capsidiol. If
12 yes, how is its biosynthesis transcriptionally regulated?

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

16

17 **Materials and Methods**

18 **Plant and fungal materials**

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

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

26

27 **RNA-seq data processing and analysis**

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

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

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

11

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

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

29

30 **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

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

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

9

10 **Quantification of scopoletin and scopolin**

11 Around 0.2 g leaf samples at 6 dpi of EV, VIGS NaEAS and VIGS NaEAH
12 plants were harvested and ground to fine powder in liquid nitrogen. The levels of
13 scopoletin and scopolin were determined by HPLC-MS/MS as described in Sun *et al.*,
14 (2014b).

15

16 **Real-Time PCR**

17 Total RNA was extracted from a 1.5 x 1.5 cm² area of leaf lamina which
18 encompassed the inoculation site with TRI reagent (Invitrogen). For each treatment,
19 4-5 replicate biological samples were collected. cDNA was synthesized from 500 ng
20 total RNA with reverse transcriptases (Thermo Scientific). Real-time PCR was
21 performed on a CFX Connect qPCR System (Bio-Rad) with iTaq Universal SYBR
22 Green Supermix (Bio-Rad) and gene-specific primers as described (Wu *et al.*, 2013).
23 For each analysis, a linear standard curve (obtained from threshold cycle number
24 versus log DNA quantity) was constructed by using a dilution series of a specific
25 cDNA sample, and the transcript levels of unknown samples were calculated
26 according to the standard curve. Finally, the relative transcript levels of target genes
27 were obtained by dividing the extrapolated transcript levels of the target genes by the
28 levels of a housekeeping gene *NaActin 2* as an internal standard from the same sample.
29 The transcript abundance of *NaActin 2* was not altered in leaves inoculated with *A.*
30 *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

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

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

12

13 **Results**

14 **Transcriptome analysis reveals the strong regulation of sesquiterpene**

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

16 Previously, we have demonstrated that, in response to *A. alternata* inoculation,
17 *N. attenuata* plants activate both jasmonate and ethylene signaling pathways to
18 regulate the biosynthesis of the phytoalexins scopoletin and its β -glycoside form,
19 scopolin (Sun *et al.*, 2014b; Li and Wu, 2016; Sun *et al.*, 2017). As *feruloyl-CoA*
20 *6'-hydroxylase 1* (*NaF6'H1*), the key enzyme gene for scopoletin biosynthesis, was
21 highly elicited at 1 day post inoculation (1 dpi) when the infection was at early stage
22 and only a few of fungal hyphae had been observed to penetrate into leaf tissues via
23 stomata (Sun *et al.*, 2014a), transcriptome analysis was performed in *A. alternata*-
24 inoculated *N. attenuata* leaves at 1 dpi to identify secondary metabolites which could
25 potentially act similarly to scopoletin. Notably, a set of genes involved in terpene
26 synthesis were strongly up-regulated, including *thiolase*, *HMG-CoA synthase*,
27 *HMG-CoA reductase*, *mevalonate kinase*, *phosphomevalonate kinase*,
28 *diphosphomevalonate decarboxylase* (*MVPP decarboxylase*),
29 *isopentenyl-diphosphate delta-isomerase*, *FPP synthase*, the capsidiol biosynthetic
30 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

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

4

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

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

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

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

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

4

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

6 To test whether capsidiol has a direct impact on fungal growth or not, we purified
7 30 mg capsidiol from 500 g of *A. alternata*-inoculated leaves, and applied this
8 compound in various concentrations to the growth medium to evaluate its inhibition
9 activity of fungal growth *in vitro*. The fungi were grown on PDA plates containing
10 with 50 µg/mL or 100 µg/mL capsidiol, and we observed fungal growth was reduced
11 to 56.6% or 43.8% of that of controls. Application of 200 µg/mL capsidiol resulted in
12 further reduction in growth to 37.1% of control (Fig. 4A, B). These results suggest
13 that capsidiol at a concentration observed *in planta* has a direct impact on the fungal
14 growth *in vitro*.

15

16 ***A. alternata*-induced capsidiol accumulation is not dependent on JA and ethylene** 17 **signaling**

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

29

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

1 **to mount a capsidiol-based defense**

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

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

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

1 and *NaEAHs* in VIGS *NaERF2*-like plants were reduced by 75% and 62%,
2 respectively (Fig. 7B, C). As expected, *A. alternata*-induced capsidiol level in VIGS
3 *NaERF2*-like plants at 3 dpi was reduced by 68% when compared with EV plants (Fig.
4 7E). However, the fungus-elicited transcriptional levels of *NaF6'H1* were not affected
5 in VIGS *NaERF2*-like plants (Fig. 7D). These results indicate that silencing
6 *NaERF2-like* substantially decreases *A. alternata*-elicited transcription of *NaEASs* and
7 *NaEAHs*, and consequently, capsidiol level, without affecting scopoletin-based
8 defense.

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

12 13 ***NaERF2-like* directly regulates the capsidiol biosynthetic gene *NaEAS12***

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

19 Several lines of evidence were consistent with the idea that *NaERF2-like* directly
20 regulates the capsidiol biosynthetic gene *NaEAS12*. When the *NaERF2-like* gene was
21 over-expressed in the protoplasts of *N. attenuata*, *luciferase* gene (*LUC*) driven by the
22 *NaEAS12* promoter showed a 5.5-fold increase in expression (Figure 8B), indicating
23 that the over-expression of *NaERF2-like* enhanced the transcriptional activity of
24 *NaEAS12* promoter. From the promoter region of *NaEAS12*, an ATCTA motif,
25 previously shown to be the binding site of RAP2.2 in *Arabidopsis* (Welsch *et al.*,
26 2007), was identified and confirmed as the binding site for the ERF2-like by
27 yeast-one-hybrid, electrophoretic mobility shift assay (EMSA) and chromatin
28 immunoprecipitation-based qPCR (ChIP-qPCR). Yeast-one-hybrid experiments
29 revealed that the ERF2-like protein could bind to the *NaEAS12* promoter fragment
30 *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

1 **Discussion**

2 Phytoalexins are important ‘chemical weapons’ employed by plants in defending
3 against pathogens. In addition to scopoletin and scopolin, two phytoalexins regulated
4 by JA and ethylene signaling pathways in response to *A. alternata* infection (Sun *et al.*,
5 2014b; Li and Wu, 2016), we demonstrate in this study that capsidiol is another
6 important phytoalexin produced by *N. attenuata*, and its biosynthesis is not dependent
7 on JA and ethylene signaling pathways but is transcriptionally regulated by a
8 transcription factor NaERF2-like.

9

10 **Capsidiol is an important phytoalexin produced in *N. attenuata* in response to *A.*** 11 ***alternata* infection**

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

24 Ideally, the benefits of a putative resistant trait should be determined in plants
25 differing only in a single gene that controls the defense trait and are otherwise
26 identical (Bergelson *et al.*, 1996). In this study, virus-induced gene silencing of
27 *NaEAHs* or *NaEASs* was used to manipulate the production of capsidiol, and the
28 results revealed that capsidiol-reduced or -depleted plants were more susceptible to *A.*
29 *alternata* (Fig. 3). In addition, capsidiol showed strong anti-fungal activities against *A.*
30 *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.

10

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). Abscisic 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

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

10

11

12 **Accession Numbers**

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

17

18 **Supplementary data:**

19 **Fig. S1. *Alternaria alternata*-elicited *NaEAS12* transcripts in EV and plants** 20 **individually silenced with the top 5 up-regulated transcription factors.**

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

27

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

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

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

4

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

7 Mean (\pm SE) relative *A. alternata*-induced *NaEIN2*, *NaF6'H1*, *NaEASs*, *NaEAHs*
8 transcripts as measured by real-time PCR in 5 replicated young leaves of EV and
9 VIGS *NaEIN2* plants at 3 dpi. Asterisks indicate levels of significant differences
10 between EV and VIGS plants with the same treatments (Student's *t*-test: *, $p < 0.05$; **,
11 $p < 0.01$; ***, $p < 0.005$)

12

13 **Table S1.**

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

17

18 **Table S2.**

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

21

22 **Table S3.**

23 Primers used in this study.

24

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3 **Figure legends**

4 **Fig. 1 The regulation of terpene biosynthetic genes in *N. attenuata* in response to** 5 ***A. alternata* inoculation at 1 day post inoculation (dpi)**

6 Genes involved in terpene synthesis were strongly regulated during transcriptome
7 analysis in 3 biological replicate samples of mock and 1 dpi: transcripts of all
8 enzymes marked in red font were up-regulated at 1 dpi, while *squalene synthase* (*SS*)
9 with blue font was down-regulated (A). To validate this regulation, relative mean
10 transcripts (\pm SE) of *HMG-CoA reductase* (A), *MVAPP decarboxylase* (B), *FPPS* (C),
11 *squalene synthase* (D), *EASs* (E) and *EAHs* (F) were measured by real-time PCR in 4
12 biological replicate 0 leaves at 1 dpi. Leaves without inoculation were collected as
13 controls (Mock 1 d). Both *EASs* and *EAHs* were detected by primers conserved in
14 gene family members. Asterisks indicate the level of significant differences between
15 Mock 1 d and 1 dpi samples (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).
16 Enzymes/substrates: 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA),
17 3-hydroxy-3-methyl-glutaryl-CoA synthase (HMG-CoA synthase),
18 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), mevalonic acid
19 kinase (MVA kinase), mevalonic acid 5-phosphate kinase (MVAP kinase), mevalonic
20 acid 5-diphosphate decarboxylase (MVAPP decarboxylase), isopentenyl diphosphate
21 (IPP), 3'3-dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl
22 pyrophosphate synthase (FPPS), farnesyl diphosphate (FPP), 5-*epi*-aristolochene (EA),
23 5-*epi*-aristolochene synthase (EAS), 5-*epi*-aristolochene hydroxylase (EAH),
24 premnaspirodiene synthase (PS), premnaspirodiene oxidase (PO), geranylgeranyl
25 diphosphate (GGPP).

26

27 **Fig. 2 Accumulation of capsidiol in 0 (young) and +3 (mature) leaves after *A.*** 28 ***alternata* inoculation**

29 Mean (\pm SE) capsidiol levels were determined by HPLC in 5 biological replicate 0
30 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'HI***
21 **transcript accumulation.**

22 Mean (\pm SE) relative *A. alternata*-induced *NaERF2-like* (A), *NaEASs* (B), *NaEAHs*
23 (C) and *NaF6'HI* (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

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

3

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

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

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

11 The asterisks indicate levels of significant differences between WT and
12 Ov-*NaERF2-like* lines after inoculation (Student's *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***,
13 $p < 0.005$). N.D., not detectable.

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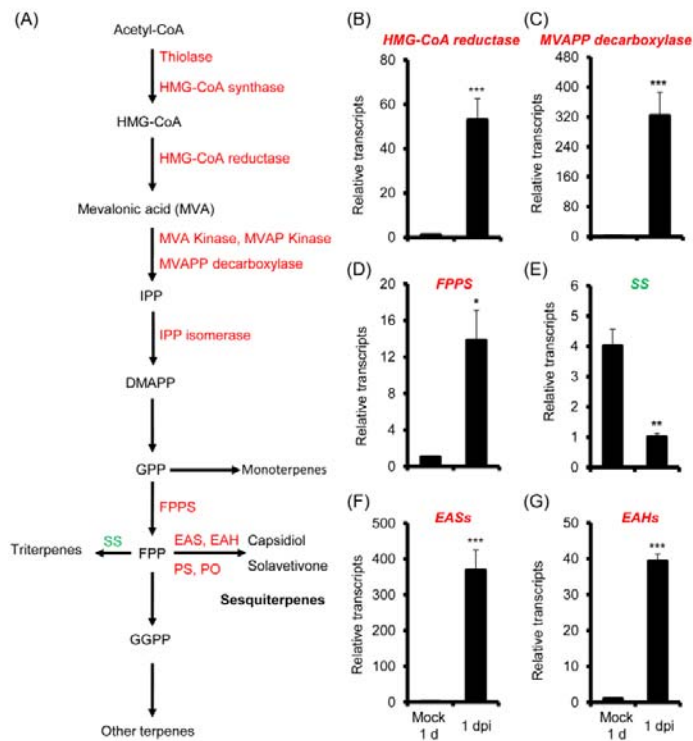
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4 **Figures**

5 **Fig. 1.**



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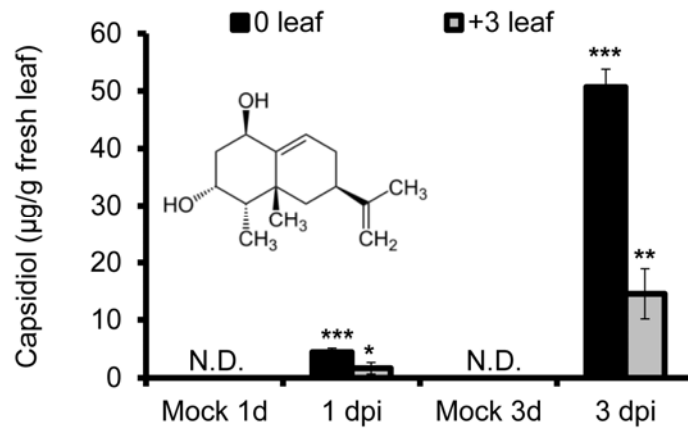
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4 **Fig. 2.**



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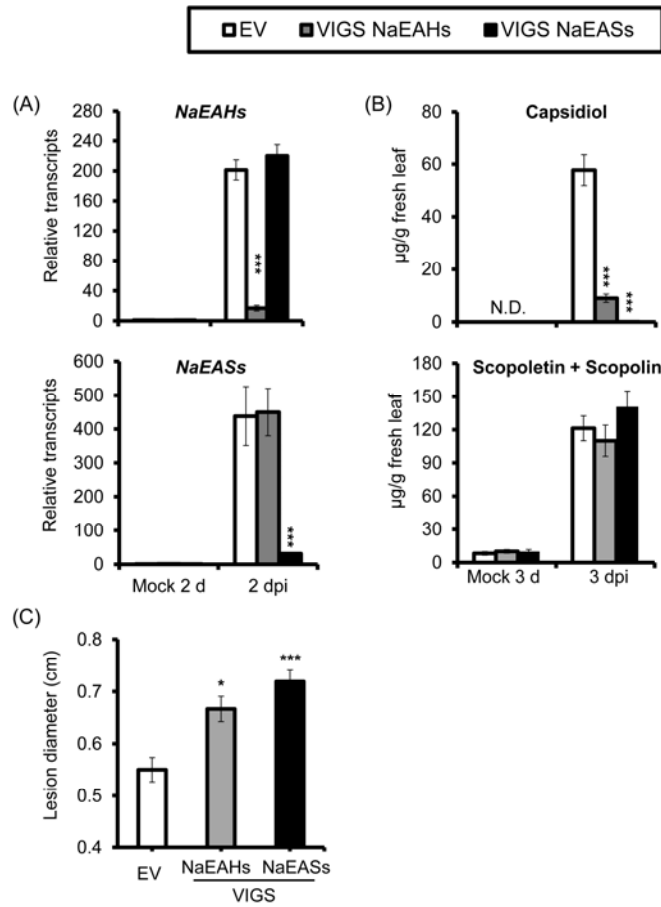
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4 **Fig. 3.**



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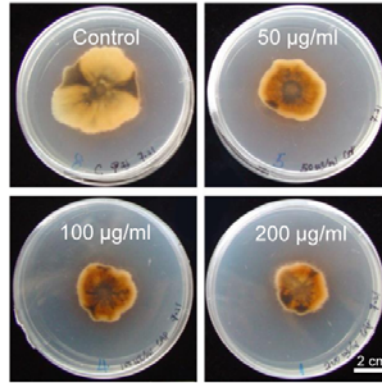
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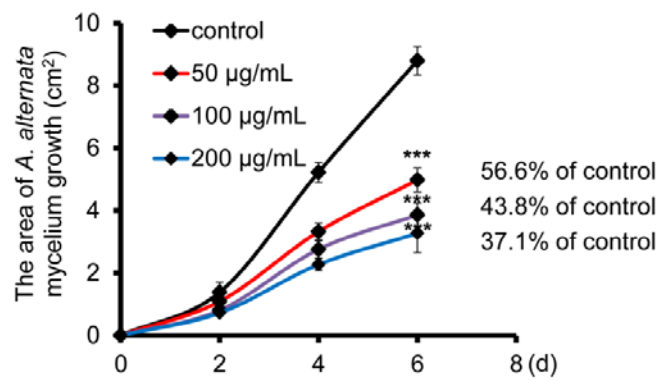
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4 **Fig. 4.**

(A)



(B)



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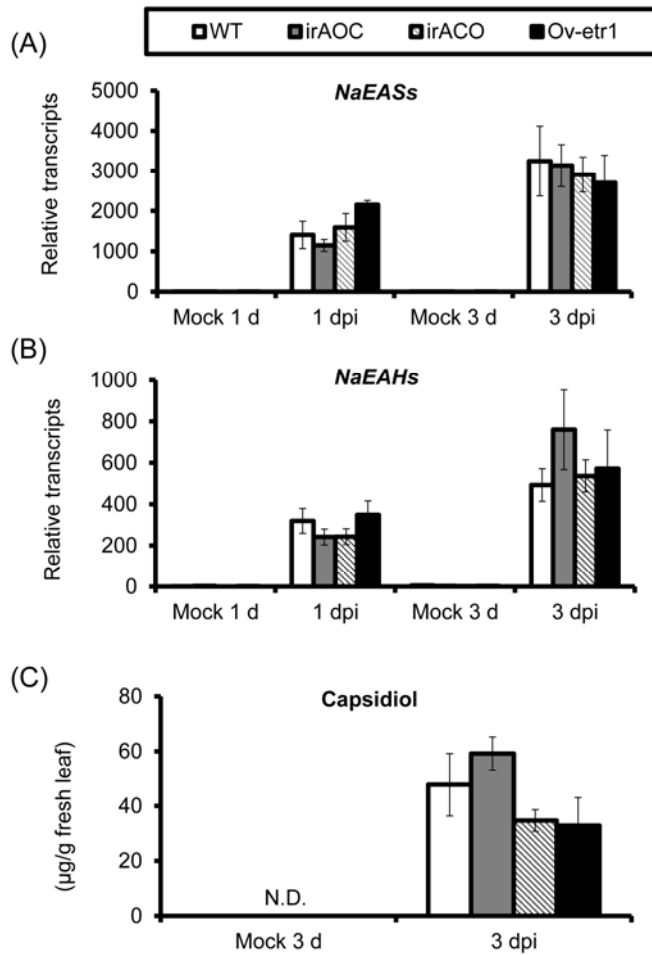
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4 **Fig. 5.**



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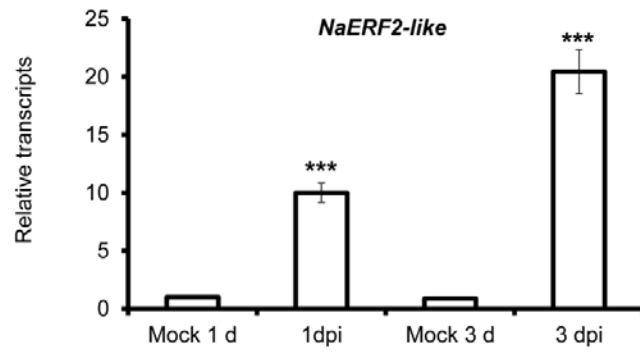
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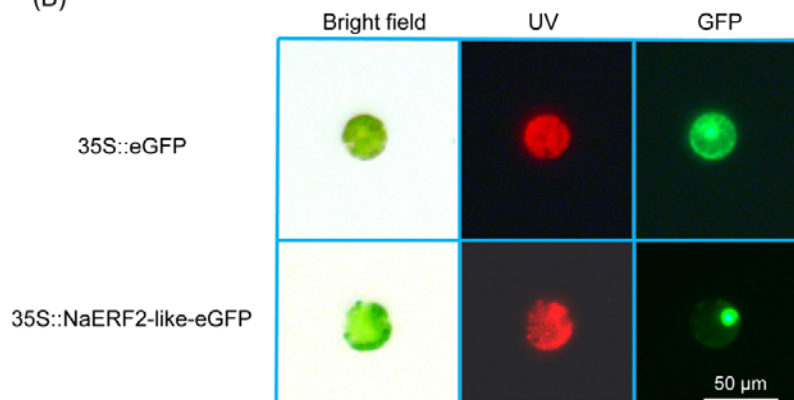
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4 **Fig. 6.**

(A)



(B)



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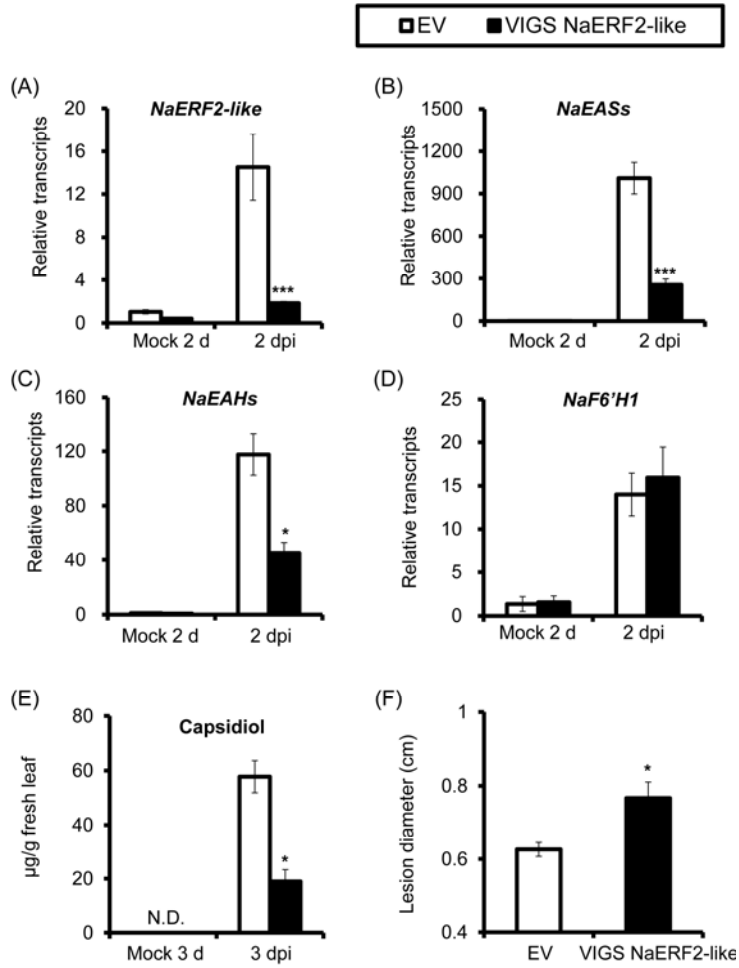
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4 **Fig. 7.**



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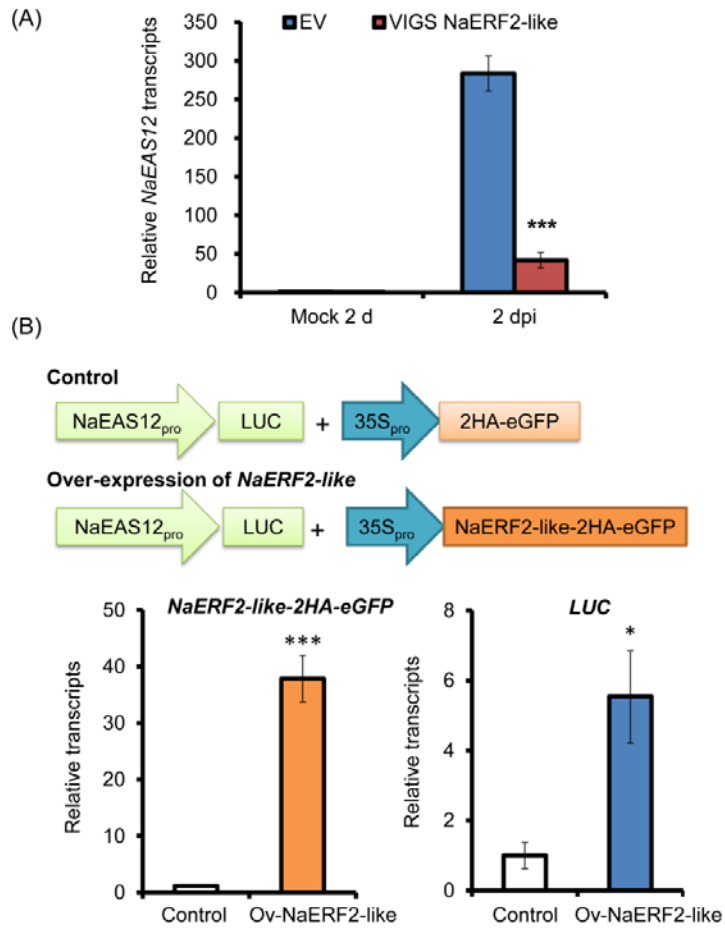
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4 **Fig. 8.**



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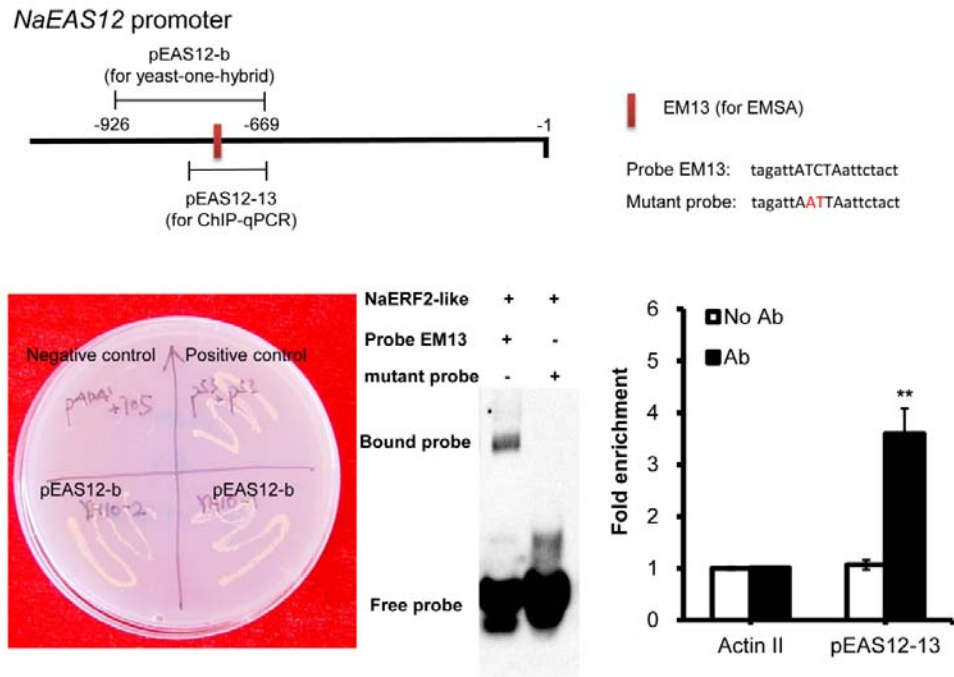
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4 **Fig. 9.**



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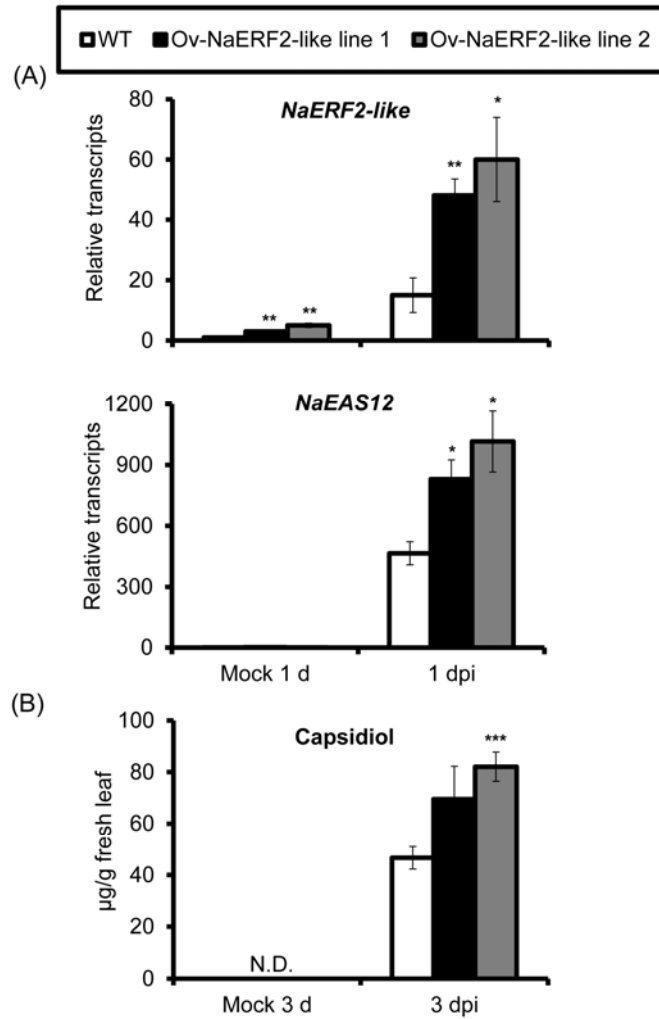
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4 **Fig. 10.**



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