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Expression profiles of genes for enzymes involved in capsidiol production in *Nicotiana benthamiana*

Authors: Soriya Rin[1], Sayaka Imano[1], Maurizio Camagna[1], Takamasa Suzuki[2], Aiko Tanaka[1], Ikuo Sato[1], Sotaro Chiba[1], Kazuhito Kawakita[1] and Daigo Takemoto[1]

Affiliation:

[1] Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-8601 Japan

[2] College of Bioscience and Biotechnology, Chubu University, Matsumoto-cho, Kasugai, Aichi, 478-8501 Japan

Corresponding author: Daigo Takemoto

E-mail: dtakemo@agr.nagoya-u.ac.jp

Telephone: +81 52 789 4029

Fax: +81 52 789 5525

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-8601 Japan

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Abstract

In Solanaceae plants, the major phytoalexins produced during the induction of plant defense are sesquiterpenoids, such as capsidiol for *Nicotiana* species and rishitin for *Solanum* species, which are produced via the mevalonate (MVA) pathway. There are eight enzymes involved in the production of farnesyl pyrophosphate (FPP), the common precursor of phytosterols for maintaining membrane integrity and sesquiterpenoid phytoalexins for plant defense. In this study, expression profiles of *N. benthamiana* genes for the production of capsidiol during the induction of disease resistance were investigated. In the genome of *N. benthamiana*, multiple copies of genes for each enzyme in the MVA pathway are identified, and the expression of some, but not all MVA genes, is significantly upregulated after inoculation with *Phytophthora infestans*, or treatment with the INF1 elicitor, a secretory protein of *P. infestans*. For genes encoding enzymes involved in the capsidiol production, 10 copies of 5-*epi*-aristolochene synthase (*NbEAS*) and 6 copies of 5-*epi*-aristolochene dihydroxylase (*NbEAH*) are identified, and all copies of them are significantly upregulated during the induction of disease resistance. Gene silencing of MAP kinase genes, *NbWIPK*, *NbSIPK* and *NbNTF4*, compromised INF1-induced production of phytoalexins. Expression analysis of control and *NbWIPK/SIPK/NTF4*-silenced plants indicated that most of the MVA genes are not under the control of these MAP kinases. In contrast, the expression pattern of *NbWIPK/SIPK/NTF4* and all copies of *NbEAH* genes showed significant correlation, suggesting that MAP kinases are critical regulators of transcriptional upregulation of specific genes for capsidiol production.

Keywords: MAP kinases, Mevalonate pathway, *Nicotiana benthamiana, Phytophthora infestans*, Sesquiterpenoid phytoalexins.

Introduction

Late blight caused by the oomycete pathogen *Phytophthora infestans* is an injurious disease for crop production. *P. infestans* can infect susceptible cultivars of crops in the genus *Solanum*, such as potato (*S. tuberosum*) and tomato (*S. lycopersicum*), and the total cost for control efforts and yield losses is estimated at multi-billion dollars annually (Fry 2008; Garelik 2002). Problems of late blight disease are most serious in developing countries where the use of fungicides is the primary strategy for disease management. Although potato cultivars resistant to this pathogen have been employed, due to selection pressure on effectors in the pathogen population, a loss of resistance among many cultivars ensued (Forbes 2012).

Most of the Solanaceous species, such as *Nicotiana* and *Capsicum* species, are resistant to *P. infestans*. To investigate the defense mechanisms of Solanaceae plants against *P. infestans*, we have conducted virus-induced gene silencing (VIGS)-based screenings for isolation of defense-related genes using *N. benthamiana*, the Solanaceous model plant (Shibata et al. 2016; Takemoto et al. 2018). From these screenings, 4 out of 7 genes for enzymes in the mevalonate (MVA) pathway were isolated as essential genes for the resistance of *N. benthamiana* against *P. infestans*. *Nicotiana* species produce several sesquiterpenoid phytoalexins, such as capsidiol, debneyol and capsidiol 3-acetate (Bailey et al. 1975; Burden et al. 1985; Uegaki et al. 1988), which are produced from farnesyl pyrophosphate (FPP), an intermediate produced via MVA pathway. Although FPP is also

70 a precursor of phytosterols for maintaining plasma-membrane integrity (Roche et al.
71 2008), silencing of MVA genes showed no detrimental effect on the growth of *N.*
72 *benthamiana* (Shibata et al. 2016), which suggested that enzymes in MVA pathway have
73 multiple copies, some of which are more dedicated to the plant pathogen defense. Our
74 screening also identified two specific genes for capsidiol production, 5-*epi*-aristolochene
75 synthase (*NbEAS*) and 5-*epi*-aristolochene dihydroxylase (*NbEAH*) as essential genes for
76 the resistance of *N. benthamiana* to *P. infestans* (Shibata et al. 2010, 2016). There are
77 multiple copies of *NbEAS* and *NbEAH* found in the genome of *N. benthamiana* (Shibata
78 et al. 2016). We have shown that gene-silencing of either *NbEAS* or *NbEAH* (using a
79 conserved sequence of all gene copies) compromised the production of capsidiol as well
80 as the resistance of *N. benthamiana* against *P. infestans*, but detailed analysis of
81 expression profiles of each copy of *NbEAS* and *NbEAH* have not been performed.
82 This study aimed to make a catalog of *N. benthamiana* genes for enzymes in the MVA
83 pathway and specific enzymes for phytoalexin production. Expression profiles of *N.*
84 *benthamiana* MVA genes, *NbEAS* and *NbEAH* were investigated during the induction of
85 defense against non-pathogenic *P. infestans* as well as after the treatment of INF1, a
86 secretory elicitor protein derived from *P. infestans*. Given that silencing of defense-related
87 mitogen-activated protein (MAP) kinases, *NbWIPK*/*SIPK*/*NTF4,* caused a significant
88 decrease of phytoalexin production, expression profiles of MVA genes, *NbEAS*, *NbEAH*
89 and other genes correlated to MAP kinases were also analyzed.
90
91 **Materials and Methods**
92 **Biological materials, growth conditions and inoculation**

93 *N. benthamiana* line SNPB-A5 (Shibata et al. 2016) was grown in an environmentally
94 controlled growth room at 23°C under a 16 h light/8 h dark per day. *P. infestans* isolate
95 08YD1 (Shibata et al. 2011) was cultured on rye media at 20°C. Inoculation of *N.*
96 *benthamiana* leaves (approx. 45 days old) with a zoospore suspension of *P. infestans* was
97 performed as previously described in Shibata et al. 2010.
98
99 **Preparation and treatment of *N. benthamiana* leaves with INF1 elicitor**
100 INF1 elicitor was prepared from *Escherichia coli* (DH5α) carrying an expression vector
101 for INF1, pFB53, as previously reported (Kamoun et al. 1997; Shibata et al. 2010). *N.*
102 *benthamiana* leaves were treated with 150 nM INF1 solution as previously described
103 (Shibata et al. 2010).
104
105 **Lactophenol trypan blue staining**
106 Leaves of *N. benthamiana* were stained with lactophenol trypan blue to visualize *P.*
107 *infestans* hyphae or dead plant cells, as described (Mizuno et al. 2019; Takemoto et al.
108 2003). Briefly, leaves of *N. benthamiana* were incubated for 2 min at 95°C in lactophenol
109 trypan blue stain (5 ml of lactic acid, 5 ml of glycerol, 5 g of phenol, and 10 mg of trypan
110 blue in 50% ethanol). After the leaves had cooled to room temperature for 1 h, the stain
111 was replaced with 1 mg/ml chloral hydrate and incubated for at least 1 day with gentle
112 shaking. Stained leaves were observed using a microscope BX51 (Olympus, Tokyo,
113 Japan).
114
115 **Virus-induced gene silencing (VIGS) of *N. benthamiana***

116 The induction of VIGS was performed as previously reported (Ratcliff et al. 2001; Shibata
117 et al. 2010). *Agrobacterium tumefaciens* GV3101 carrying the binary TRV RNA 1
118 construct pBINTRA6, and the TRV RNA2 vector pTV00 or pTV00-W/S (Ohtsu et al.
119 2014), were cultured to saturation in Luria-Bertani (LB) media. Bacterial suspensions
120 were then collected by centrifugation at 16,000 x g for 1 min. The bacterial cells were
121 then resuspended in 10 mM MES-NaOH (pH 5.6), 10 mM $MgCl_2$ and 150 μM
122 acetosyringone (final OD_{600} = 0.5) and incubated at room temperature for 2 h. The
123 cultures were mixed in a 1:1 ratio (RNA1/RNA2), to infiltrate leaves of *N. benthamiana*
124 using a syringe without a needle. After 3-4 weeks of infiltration, upper leaves of the
125 inoculated plants were used for experiments.
126
127 **Extraction and quantification of phytoalexins**
128 *N. benthamiana* leaves (50 mg) treated with water or 150 nM INF1 were washed in ethyl
129 acetate/cyclohexane (1:1) for 1 h with gentle shaking. Phytoalexins extracted in the
130 organic solvent were quantified by GC/MS using an Agilent Technologies 7890A GC
131 System using a DuraBond Ultra Inert column (length 30 m; diameter 0.25 mm; film 0.25
132 μm, Agilent Technologies, Santa Clara, CA, USA) as previously described (Camagna et
133 al. 2020). Purified capsidiol, debneyol and capsidiol-3-acetate (Matsukawa et al. 2013)
134 were used for quantitative analysis.
135
136 **Extraction of total RNA and RNA-seq analysis**
137 For the RNA isolation, 100 mg fresh weight of *N. benthamiana* leaves was collected and
138 frozen in liquid nitrogen, then store at -80 ˚C until use. The frozen leaves were ground

with PowerMasher II (Nippi, Tokyo, Japan), and the total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Quality and quantity of isolated RNA were evaluated using Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA was purified with NEBNext Poly(A) mRNA magnetic isolation module (New England Biolabs, Ipswich, MA, USA) and used for the construction of cDNA libraries using the NEBNext Ultra II RNA library prep kit for Illumina and NEBNext Multiplex oligos for Illumina (New England Biolabs) according to the manufacturer's instructions. RNA-Seq libraries were sequenced using Illumina NextSeq 500 (Illumina, San Diego, CA, USA) with single read mode. The nucleotides of each read with less than 13 quality value were masked and reads less than 50 bp in length were discarded before mapping. The filtered reads were mapped to annotated cDNA sequences for *N. benthamiana* (Niben101_annotation.transcripts, Sol genomics, https://solgenomics.net) and *P. infestans* (ASM14294v1, Broad Institute, https://www.broadinstitute.org) using Bowtie software (Langmead et al. 2009) and the number of reads mapping to each annotated cDNA was counted. For each gene, the relative fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated and significant difference from the control (*p* values) was assessed by the two-tailed Student's *t* test. RNA-seq data reported in this work are available in GenBank under the accession numbers SAMD00204668-70 and SAMD00204740-57.

Results and Discussion

Expression profiles of *N. benthamiana* genes for enzymes in the MVA pathway during the defense induction against *P. infestans*

We first made a list of genes for enzymes in the MVA pathway in the genome sequence of *N. benthamiana* (ver. 1.0.1, Bombarely et al. 2012). A list of all genes for enzymes in the MVA pathway was compiled, based on the set of genes which was previously found to be required for resistance in *N. benthamiana* against *P. infestans* as well as homolog sequences to *A. thaliana* MVA pathway genes (Supplementary Table S1). Given that *N. benthamiana* has an allopolyploid genome (Goodin et al. 2008), two highly homologous genes were frequently found in previous studies (Matsukawa et al., 2013; Ohtsu et al. 2014; Shibata et al. 2016), and they are expected to derive from the two *Nicotiana* species ancestral to *N. benthamiana*. For example, a set of highly homologous genes for acetyl-CoA thiolase (ACAT) are designated as *NbACAT1a* and *NbACAT1b.*

To investigate the expression profiles of MVA genes during the induction of plant defense, RNA-seq analysis was performed for *N. benthamiana* leaves inoculated with *P. infestans* isolate 08YD1 (non-pathogenic to *N. benthamiana*), or treated with the elicitor INF1, a secretory protein of *P. infestans* (Kamoun et al. 1997). For leaves inoculated with the pathogen, the abundance of *P. infestans* was estimated from the ratio of reads that mapped to cDNA sequences of *P. infestans*, relative to the reads that mapped to cDNA sequences of *N. benthamiana* (Supplementary Fig. S1). Just after the inoculation of zoospore suspension, the ratio of *P. infestans* reads was initially 0.5%, but significantly increased to approx. 6 % at 24 h post inoculation (hpi), reflecting an increase in biomass. Hyphal growth of *P. infestans* was observed at 24 hpi, but cell death of *N. benthamiana* became evident at 48 h, which is consistent with slightly decreased biomass of *P. infestans* at 48 h (Supplementary Fig. S1).

184 The MVA pathway begins with 2 units of acetyl-CoA converted into acetoacetyl CoA
185 by ACAT (Fig. 1). In *N. benthamiana*, 5 copies of *NbACAT* genes can be identified, and
186 are designated as *NbACAT1a, NbACAT1b, NbACAT2a, NbACAT2b*, and *NbACAT3*.
187 Expression of *NbACAT1a* and *NbACAT1b* are highly upregulated in leaves inoculated
188 with *P. infestans* at 24 hpi and decreased at 48 hpi. Treatment of INF1 also enhanced the
189 expression of *NbACAT1a* and *NbACAT1b,* but not other *NbACAT* genes (Fig. 1). The
190 second step of the MVA pathway is the reaction in which acetyl-CoA condenses with
191 acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by
192 HMG-CoA synthase (HMGS). Among 5 copies of *NbHMGS* genes, *NbHMGS1a* and *1b*
193 showed a significant increase of expression in responses to *P. infestans* and INF treatment,
194 while *NbHMGS2a* and *2b* showed relatively little increase (Fig. 1). The third reaction of
195 the MVA pathway is catalyzed by HMG-CoA reductase (HMGR), in which HMG-CoA
196 is reduced to mevalonic acid by NADPH. HMGR is known as the rate-controlling enzyme
197 of the mevalonate pathway, regulated on multiple levels, including transcription,
198 translation, degradation and phosphorylation (Burg and Espenshade 2011; Stermer et al.
199 1994). Expression of *NbHMGR2* is highly upregulated by the inoculation with *P. infestans*
200 and treatment with INF1, while a slight induction of *NbHMGR1a* and *1b* genes was
201 observed in response to the inoculation with *P. infestans* and treatment with INF1 (Fig.
202 1). Mevalonate is then phosphorylated at the 5-OH position by mevalonate-5-kinase
203 (MVK), and further phosphorylated by phosphomevalonate kinase (PMVK) to yield
204 mevalonate-5-pyrophosphate (Fig. 1). There are two homologous genes for *NbMVK* and
205 *NbPMVK*, and the expression of both copies is upregulated by the inoculation with *P.*
206 *infestans* and treatment with INF1. Similarly, there are two copies of genes for

mevalonate-5-pyrophosphate decarboxylase (MVD), which catalyze the decarboxylation of mevalonate-5-pyrophosphate to yield isopentenyl pyrophosphate (IPP). Both copies of NbMVD are upregulated during the induction of plant defense (Fig. 1). The last enzyme for the MVA pathway is IPP isomerase (IPPI), which catalyzes the conversion of IPP to dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are then condensed into farnesylpyrophosphate (FPP), the precursor of phytosterol, triterpenes and sesquiterpenes, by FPP synthase (FPPS) (Fig. 1). Transcription of 4 copies of *NbIPPI* genes is significantly upregulated by the inoculation with *P. infestans* and treatment with INF1. Similarly, 4 copies of *NbFPPS* genes are upregulated during the induction of plant defense (Fig. 1).

Expression profile of *N. benthamiana* genes for enzymes specifically involved in the production of phytoalexins

FPP produced via the MVA pathway is the precursor of sesquiterpenoid phytoalexins (Fig. 2). Formation of capsidiol, the major phytoalexin of *N. benthamiana*, involves the cyclization of FPP to the sesquiterpenoid hydrocarbon 5-*epi*-aristolochene catalyzed by 5-*epi*-aristolochene synthase (EAS) (Facchini and Chappell 1992), and two subsequent hydroxylations by a cytochrome P450, 5-*epi*-aristolochene dihydroxylase (EAH) (Ralston et al. 2001). 5-*epi*-aristolochene is also the precursor of debneyol, but the enzyme(s) involved in debneyol production have not been identified. There are 10 copies of *NbEAS* and 6 copies of *NbEAH* genes in the genome of *N. benthamiana* (Supplementary Table S2), and all copies of *NbEAS* and *NbEAH* are significantly upregulated by the inoculation with *P. infestans* and treatment with INF1 (Fig. 2).

WIPK, SIPK and NTN4 are involved in the production of sesquiterpenoid phytoalexins in *N. benthamiana*

Mitogen-activated protein (MAP) kinases have essential roles in both basal and *R* gene-dependent disease resistance. *N. tabacum* wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) or their orthologs in other plant species are known to be involved in the defense against a wide range of pathogens (Nakagami et al. 2005; Ren et al. 2008; Seo et al. 1995; Tanaka et al. 2009; Zhang and Klessig et al. 1997, 1998). In *N. benthamiana*, WIPK, SIPK and NTF4 (closest homolog of SIPK) are activated by their upstream MAP kinase kinase MEK2 and functionally redundant for the activation of downstream WRKY transcription factors (Ishihama et al. 2011). *Arabidopsis thaliana* orthologues of WIPK and SIPK (MPK3 and MPK6) together regulate the production of the indole phytoalexin, camalexin (Ren et al. 2008).

To investigate the involvement of defense-related MAP kinases in the production of sesquiterpenoid phytoalexins in *N. benthamiana*, *NbWIPK/SIPK/NTF4*-silenced plants were generated. There are 2 copies of *NbWIPK* and *NbSIPK*, respectively, and a single *NbNTF4* gene in the *N. benthamiana* genome, and in *NbWIPK/SIPK/NTF4*-silenced plants, these genes were significantly downregulated in both water-treated and INF1-treated leaves (Fig. 3a). Analysis using the SGN VIGS tool (Fernandez-Pozo et al. 2015) confirmed that the construct pTV00-W/S specifically targets *NbWIPK/SIPK/NTF4* genes, and no potential off-target effect was detected. As previously reported (Ishihama et al. 2011), gene silencing of *NbWIPK/SIPK/NTF4* compromised resistance against *P. infestans* (Fig. 3b), while specific silencing of neither *NbWIPK* nor *NbSIPK/NTF4*

253 compromised resistance of *N. benthamiana* to *P. infestans* (data not shown). Production
254 of sesquiterpenoid phytoalexins, capsidiol, debneyol and capsidiol 3-acetate, was induced
255 by INF1 treatment, and gene silencing of *NbWIPK/SIPK/NTF4* significantly reduced the
256 production of these phytoalexins, indicated that these MAP kinases are involved in the
257 activation of phytoalexin production in *N. benthamiana* (Fig. 3c).
258
259 **_N. benthamiana_ WIPK, SIPK and NTN4 are involved in the transcriptional**
260 **upregulation of _EAH_ genes**
261 To identify genes for phytoalexin production under the control of these MAP kinases, we
262 conducted the Pearson correlation coefficient (PCC) analysis between expression profiles
263 of *NbWIPK/SIPK/NTF4* genes and genes for enzymes in the MVA pathway using RNA-
264 seq data of control and *NbWIPK/SIPK/NTF4*-silenced *N. benthamiana* (Fig. 4 and
265 Supplementary Fig. S2). For most of the genes in the MVA pathway, no statistically
266 significant effect of *NbWIPK/SIPK/NTF4*-silencing was detected in *N. benthamiana*
267 leaves treated with INF (Supplementary Fig. S2). Among 29 MVA genes, 6 genes showed
268 a significant decrease by gene silencing of *NbWIPK/SIPK/NTF4* (Supplementary Fig. S2).
269 However, only *NbHMGR1a* and *NbHMGR1b*, but not the other 4 *NbHMGR* genes,
270 showed a significant correlation with expression of *NbWIPK/SIPK/NTF4* by PCC
271 analysis (Fig. 4 and Supplementary Fig. S2). Given that expression levels of *NbHMGR1a*
272 and *NbHMGR1b* are only 15.2 and 6.2% of *NbHMGR2*, respectively, overall expression
273 of *NbHMGR* was not significantly affected by these MAP kinases (Fig. 4). These data
274 thus indicated that genes for enzymes in the MVA pathway are not under the strong
275 control of the MAP kinases silenced in these plants.

Although all 10 *NbEAS* genes tended to show decreased expression in *NbWIPK/SIPK/NTF4*-silenced plants, only 4 of them were statistically significantly downregulated by their gene silencing. Significant positive correlation with *NbWIPK/SIPK/NTF4* was detected only for *NbEAS1* and *NbEAS2* by PCC analysis (Supplementary Fig. S3). In contrast, expression of all 6 *NbEAH* genes is significantly downregulated by gene silencing of *NbWIPK/SIPK/NTF4*, and expression pattern of all *NbEAH* genes showed close correlation with that of *NbWIPK/SIPK/NTF4* (Fig. 4). These results indicate that expression of *NbEAH* genes is regulated by these MAP kinases.

While the production of phytoalexins was drastically reduced in *NbWIPK/SIPK/NTF4*-silenced plants (Fig. 3), reduction in the expression of genes for enzymes in MVA pathway, *NbEAS* and *NbEAH* was relatively moderate (Fig. 3, Supplementary Figs. S2 and S3). Given that previous studies suggested the possible involvement of post-transcriptional regulations in plant disease resistance (e.g. Mizuno et al. 2019; Stermer et al. 1994), these defense related MAP kinases might regulate translation, activation or stability of enzymes for phytoalexin production.

Identification of genes closely correlated with WIPK, SIPK and NTN4 in *N. benthamiana*

Pearson correlation coefficient (PCC) analysis was applied to all *N. benthamiana* genes to identify defense-related genes, whose expression is affected by gene silencing of *NbWIPK/SIPK/NTF4*. Among 57,139 annotated *N. benthamiana* genes, 1,387 (2.4 %) genes showed significantly increased expression by INF1 treatment at 24 h (LogFC > 3, compared with water treatment). They were selected to be analyzed for their correlation

299 with the expression profiles of *NbWIPK/SIPK/NTF4*. Close correlation (*r* value > 9.0)
300 was detected for 72 genes (5.2% of highly upregulated genes) (Supplementary Table S3).
301 This set of genes contains 7 genes associated to ethylene, such as genes required for the
302 biosynthesis of ethylene, namely 1-aminocyclopropane-1-carboxylate (ACC) synthase
303 and ACC oxidase, as well as ethylene-responsive transcription factors (ERF)
304 (Supplementary Table S3 and Fig. S4), suggesting that activation of ethylene signaling is
305 under the control of these defense-related MAP kinases. This result is consistent with our
306 previous finding, showing that gene silencing of *NbWIPK/SIPK/NTF4* caused the
307 reduction of ethylene production in *N. benthamiana* leaves treated with INF1 (Ohtsu et
308 al. 2014). Genes correlated with *NbWIPK/SIPK/NTF4* also included genes encoding E3
309 ubiquitin-protein ligases, U-box domain-containing proteins, RING-H2 finger proteins
310 and BTB/POZ domain-containing protein (Supplementary Table S3), suggesting that
311 ubiquitination-mediated protein degradation, which is known as a key regulatory
312 mechanisms for different steps of plant immune responses (Furlan et al. 2012), is also
313 under the control of NbWIPK/SIPK/NTF4. Besides genes for ERF, genes for various
314 types of transcription factors or regulatory proteins are listed, including MYB
315 transcription factors, Cys2/His2-type zinc-finger transcription factors and plant-specific
316 VQ motif-containing proteins (Supplementary Table S3). VQ motif-containing proteins
317 interact with WRKY transcription factors, and by doing so, play either positive or
318 negative roles in SA- and/or JA-mediated plant immune responses (Jing and Lin 2015).
319 These results indicated that NbWIPK/SIPK/NTF4 play a key role as upstream regulators
320 of multiple layers of transcriptional regulation for defense-related genes.
321

Concluding remarks

In this study, *N. benthamiana* genes of enzymes for capsidiol production are listed and their expression profiles during the induction of defense against *P. infestans* are investigated. Expression of *NbEAH* genes, encoding enzymes dedicated to capsidiol production, is under the control of NbWIPK/SIPK/NTF4, while the upregulation of genes for the MVA pathway is likely to be controlled by different mechanisms. Given that the MVA pathway is involved in the production of phytoalexins in Solanaceae species, which do not occur in most other plants, the mechanism for the upregulation of MVA genes during the induction of disease resistance should have evolved after the separation of the Solanaceae lineage. Consistently, in *A. thaliana*, which produces indole phytoalexins, genes for the MVA pathway are not upregulated when challenged by various pathogens, based on our investigation on the expression profiles of MVA genes in Arabidopsis eFP Browser (Winter et al. 2007) (data not shown).

NbEAS and *NbEAH* are specific genes for plant species that produce capsidiol, and their copy number as well as relative expression level, is higher than for MVA genes (Supplementary Table 2 and Fig. 2). *NbEAS1* (Niben101Scf07725g01004.1) and *NbEAS2* (Niben101Scf07725g00004.1), or *NbEAS3* (Niben101Scf03993g06006.1) and *NbEAS4* (Niben101Scf03993g05005.1) are neighboring genes in *N. benthamiana* genome, and some pseudogenes of *NbEAS* are also found near *NbEAS1* and *NbEAS2* (data not shown). Similarly, 3 *NbEAH* genes (*NbEAH1*, Niben101Scf00072g06001.1, *NbEAH3*, Niben101Scf00072g05003.1 and *NbEAH4*, Niben101Scf00072g05004.1) are found in same contig in the draft genome of *N. benthamiana*, indicating that gene duplication events were involved in increasing the number of *NbEAS* and *NbEAH* genes in *N.*

345 *benthamiana.* These lineage-specific duplications of the *NbEAS and NbEAH* genes
346 suggest the emergence of a specialized pathway during their evolution (Panchy et al.
347 2016); thus, the mechanisms for the regulation of these genes probably developed
348 independently from that of the MVA pathway genes. A recent study indicated that WIPK
349 and SIPK are involved in the expression of MVA genes, *EAS* and *EAH* by wounding in
350 *N. tabacum* (Kojima et al. 2019). Further analysis with additional data sets for different
351 time point after elicitor treatment or different stimuli in *N. benthamiana* and related
352 species, would help to group genes, that are regulated by the same transcriptional
353 mechanisms, and thereby contribute to finding new promoter motifs and related
354 transcription factors crucial for the regulation of phytoalexin production.
355
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366
367 **Compliance with ethical standards**

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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481

482 **Figure legends**

483 **Fig. 1** Expression profiles of *Nicotiana benthamiana* genes encoding enzymes in the
484 mevalonate pathway. Gene expression (FPKM value) was determined by RNA-seq
485 analysis of *N. benthamiana* leaves treated with water (H_2O), 150 nM INF1 for 24 h or
486 inoculated with *Phytophthora infestans*. Data are means ±SE (n = 3). Data marked with
487 asterisks are significantly different from control as assessed by the two-tailed Student's *t*
488 test: **P<0.01. ACAT, Acetoacetyl-CoA thiolase; HMGS, Hydroxymethylglutaryl-CoA
489 synthase; HMGR, Hydroxymethylglutaryl-CoA-reductase; MVK, Mevalonate-5-kinase;
490 PMVK, Phosphomevalonate kinase; MVD, Mevalonate-5-pyrophosphate decarboxylase;
491 IPPI, Isopentenyl pyrophosphate isomerase; FPPS, Farnesyl pyrophosphate synthase.

492

493 **Fig. 2** Expression profiles of *Nicotiana benthamiana* genes encoding specific enzymes
494 for phytoalexin production. The gene expression (FPKM value) was determined by RNA-
495 seq analysis of *N. benthamiana* leaves treated with water, 150 nM INF1 for 24 h, or
496 inoculated with *Phytophthora infestans*. Data are means ±SE (n = 3). Data marked with
497 asterisks are significantly different from control as assessed by the two-tailed Student's *t*
498 test: **P<0.01. EAS, 5-*epi*-aristolochene synthase; EAH, 5-*epi*-aristolochene-1,3-
499 dihydroxylase.

500

501 **Fig. 3** *Nicotiana benthamiana* MAP kinases WIPK, SIPK and NTF4 are involved in the
502 INF1-induced production of sesquiterpenoid phytoalexins. **a** Expression of *Nicotiana*
503 *benthamiana WIPK*, *SIPK* and *NTF4* genes (FPKM value) in control (TRV) and

504 TRV:W/S/N-inoculated *N. benthamiana* leaves 24 h after treatment with water or 150 nM
505 INF1 analyzed by RNA-seq. Data are means ±SE (n = 3). Data marked with asterisks are
506 significantly different from control as assessed by the two-tailed Student's *t* test: **P <
507 0.01. **b** Control (TRV) and *NbWIPK/SIPK/NTF4*-silenced *N. benthamiana* were
508 inoculated with *Phytophthora infestans* and disease symptoms were photographed 9 days
509 after inoculation. Appearance of disease symptoms was categorized into 5 classes
510 according to the severity of disease symptoms. 0, no visible symptom; 1, small wilted
511 spots in inoculated area; 2, browning <50% of the inoculated side of leaves; 3, browning
512 >50% of the inoculated side of leaves; 4, development of disease symptoms over central
513 leaf vein. Plot showing percentage of *N. benthamiana* leaves with disease symptom
514 severities from 3, 6 and 9 days post inoculation (dpi). Data marked with asterisks are
515 significantly different from control as assessed by one-tailed Mann-Whitney U tests: *P
516 < 0.05. **c** Sesquiterpenoid phytoalexins were extracted from leaves of control (TRV) and
517 *WIPK/SIPK/NTF4*-silenced *N. benthamiana* 24 h after 150 nM INF1 treatment and
518 quantified using GC/MS. Data are means ±SE (n = 4). Note that no production of
519 phytoalexins was detected for water-treated samples. Data marked with asterisks are
520 significantly different from control as assessed by the two-tailed Student's *t* test: **P <
521 0.01.
522
523 **Fig. 4** Expression profiles of *Nicotiana benthamiana* genes encoding
524 hydroxymethylglutaryl-CoA-reductase (HMGR) and 5-*epi*-aristolochene-1,3-
525 dihydroxylase (EAH) in *NbWIPK/SIPK/NTF4*-silenced plants. Gene expression (FPKM
526 value) was determined by RNA-seq analysis of TRV-inoculated control (TRV) or

527 *NbWIPK/SIPK/NTF4*-silenced (TRV:W/S/N) *N. benthamiana* leaves treated with water
528 (H_2O) or 150 nM INF1 for 24 h. Data are means ±SE (n = 3). Data marked with asterisks
529 are significantly different from TRV-inoculated control as assessed by the two-tailed
530 Student's *t* test: *P<0.05, **P<0.01. Results of Pearson correlation coefficient analysis
531 are shown above columns where expression patterns showed significant positive
532 correlation ($r > 0.8$, $p < 0.05$) with that of *NbWIPK/SIPK/NTF4.* Examples of PCC
533 analysis (for *NbHMGR2* and *NbEAH5*) are shown in the right graphs.

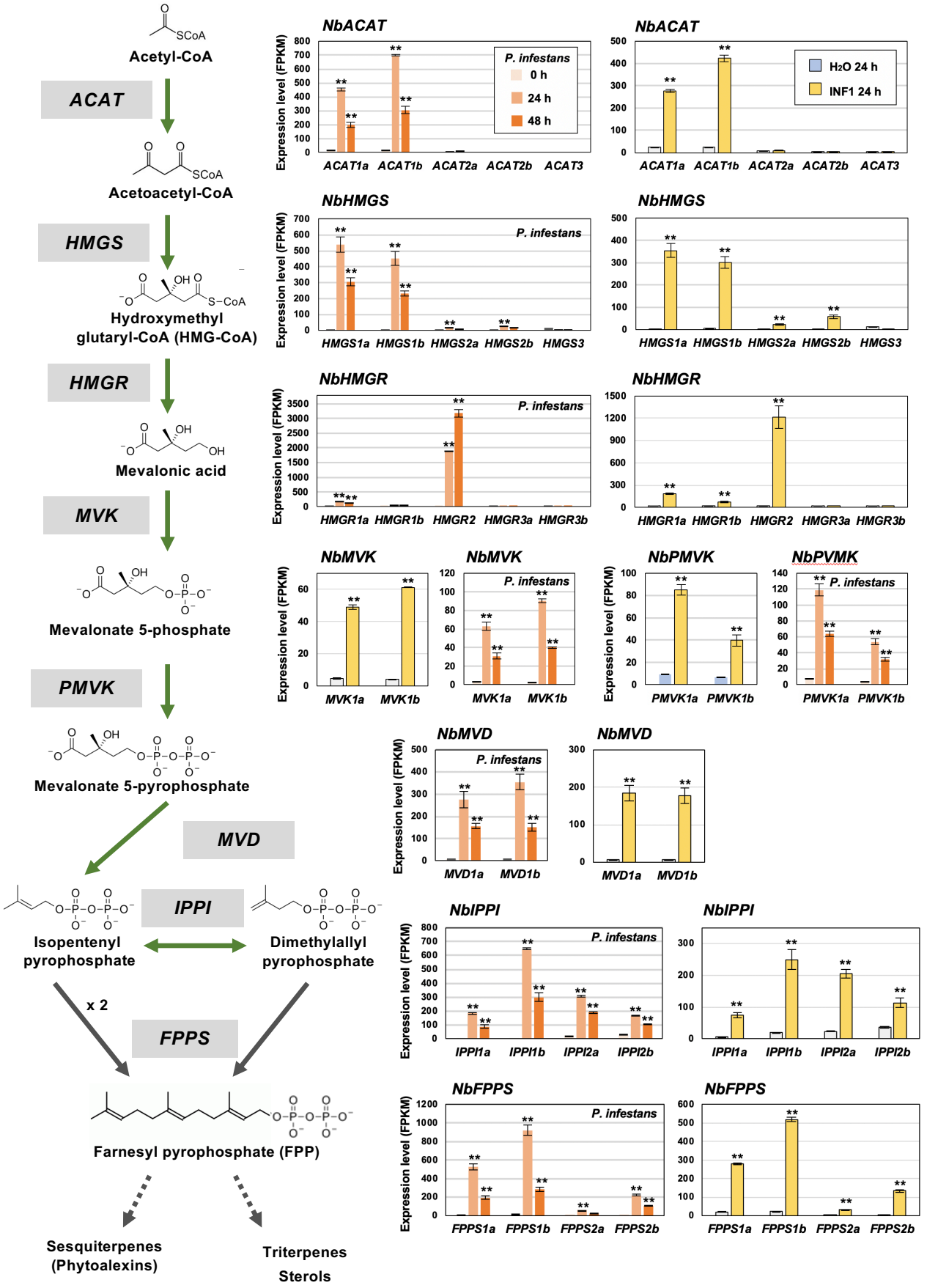


Fig. 1 Expression profiles of *Nicotiana benthamiana* genes encoding enzymes in mevalonate pathway. Gene expression (FPKM value) was determined by RNA-seq analysis of *N. benthamiana* leaves treated with water (H_2O), 150 nM INF1 for 24 h or inoculated with *Phytophthora infestans*. Data are means ± SE (n = 3). Data marked with asterisks are significantly different from control as assessed by the two-tailed Student's *t* test: **P<0.01. ACAT, Acetoacetyl-CoA thiolase; HMGS, Hydroxymethylglutaryl-CoA synthase; HMGR, Hydroxymethylglutaryl-CoA-reductase; MVK, Mevalonate-5-kinase; PMVK, Phosphomevalonate kinase; MVD, Mevalonate-5-pyrophosphate decarboxylase; IPPI, Isopentenyl pyrophosphate isomerase; FPPS, Farnesylpyrophosphate synthase.

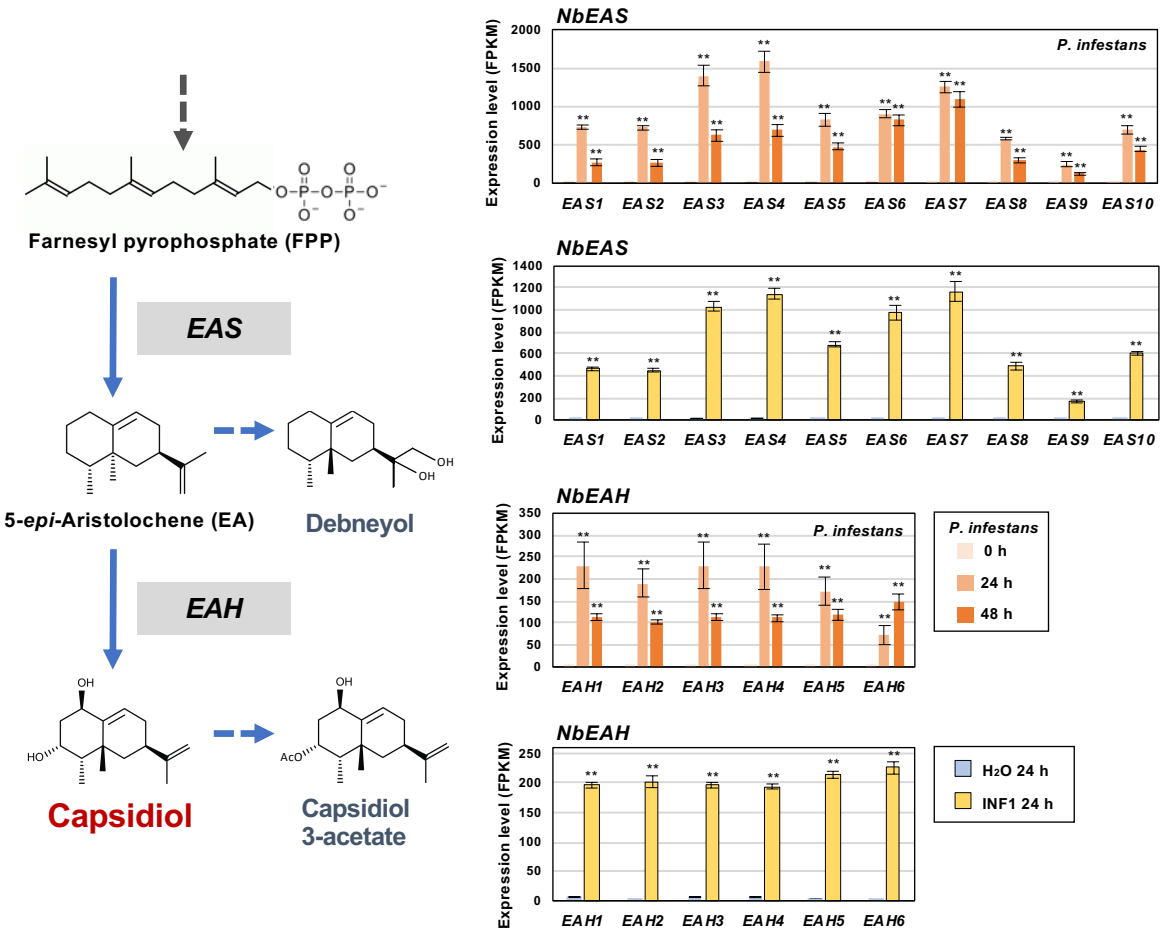


Fig. 2 Expression profiles of *Nicotiana benthamiana* genes encoding specific enzymes for phytoalexin production. The gene expression (FPKM value) was determined by RNA-seq analysis of *N. benthamiana* leaves treated with water, 150 nM INF1 for 24 h or inoculated with *Phytophthora infestans*. Data are means ±SE (n = 3). Data marked with asterisks are significantly different from control as assessed by the two-tailed Student's *t* test: **P<0.01. EAS, 5-*epi*-aristolochene synthase; EAH, 5-*epi*-aristolochene-1,3-dihydroxylase.

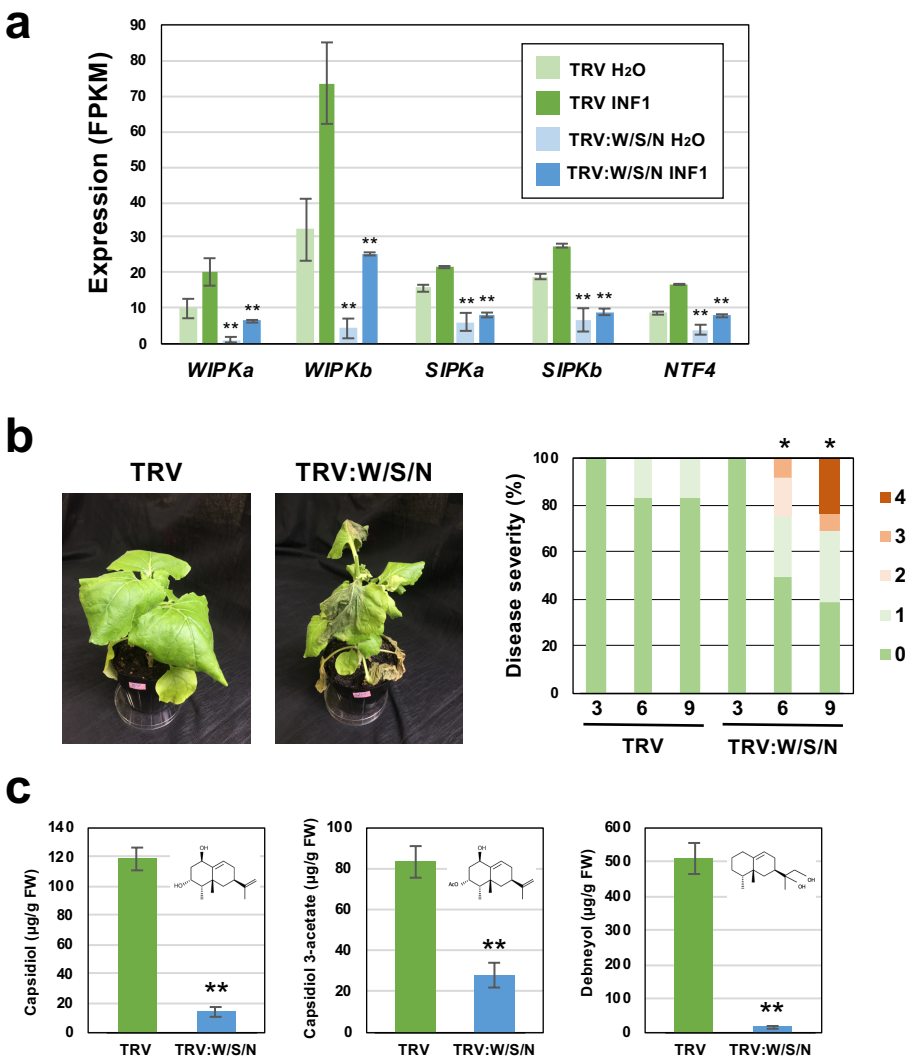


Fig. 3 *Nicotiana benthamiana* MAP kinases WIPK, SIPK and NFF4 are involved in the INF1- induced production of sesquiterpenoid phytoalexins.

a Expression of *Nicotiana benthamiana WIPK*, *SIPK* and *NTF4* genes in control (TRV) and TRV:W/S/N-inoculated *N. benthamiana* leaves 24 h after treatment with water or 150 nM INF1. Data are means ± SE (n = 3). Data marked with asterisks are significantly different from control as assessed by the two-tailed Student's *t* test: **P < 0.01.

b Control (TRV) and *WIPK/SIPK/NTF4*-silenced *N. benthamiana* were inoculated with *Phytophthora infestans* and disease symptoms were photographed 9 days after inoculation. Appearance of disease symptoms was categorized into 5 classes according to the severity of disease symptoms. 0, no visible symptom; 1, small wilted spots in inoculated area; 2, browning <50% of inoculated side of leaf; 3, browning >50% of inoculated side of leaf; 4, development of disease symptoms over central leaf vein. Plot showing percentage of *N. benthamiana* leaves with disease symptom severities from 3 to 5 days post inoculation (dpi). Data marked with asterisks are significantly different from control as assessed by one-tailed Mann-Whitney U tests: *P < 0.05.

c Sesquiterpenoid phytoalexins were extracted from leaves of control (TRV) and *WIPK/SIPK/NTF4*-silenced *N. benthamiana* 24 h after 150 nM INF1 treatment and quantified using GC/MS. Data are means ±SE (n = 4). Note that no production of phytoalexins was detected for water-treated samples. Data marked with asterisks are significantly different from control as assessed by the two-tailed Student's *t* test: **P < 0.01.

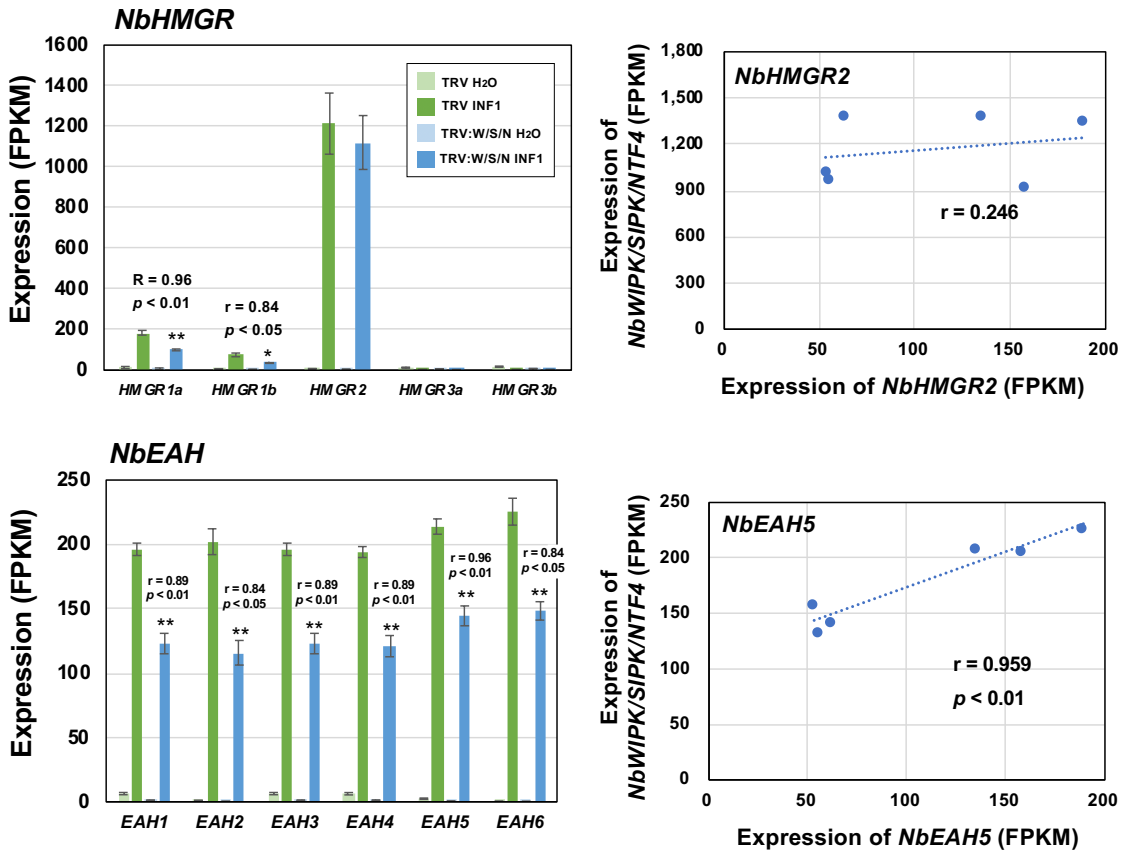
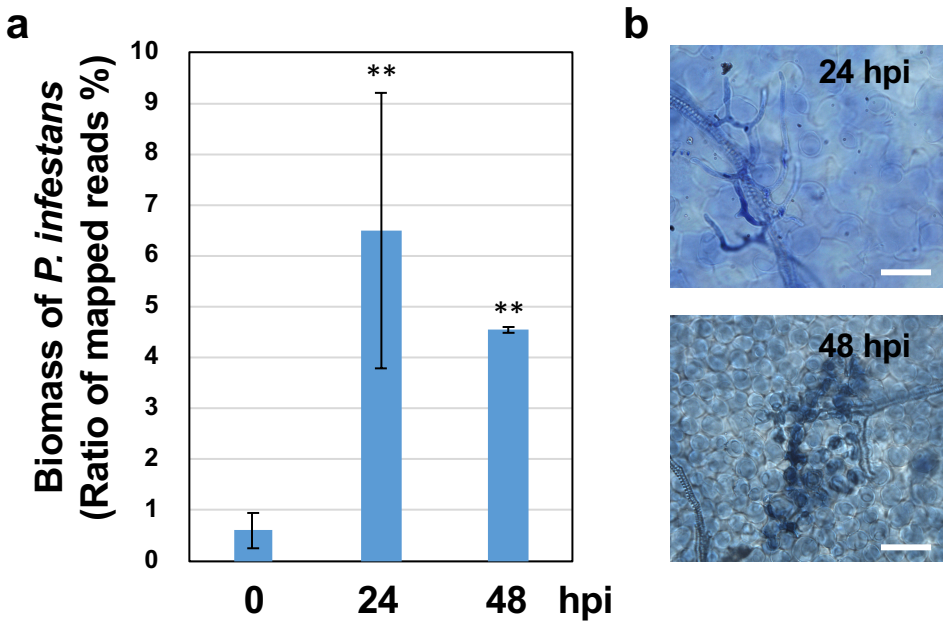
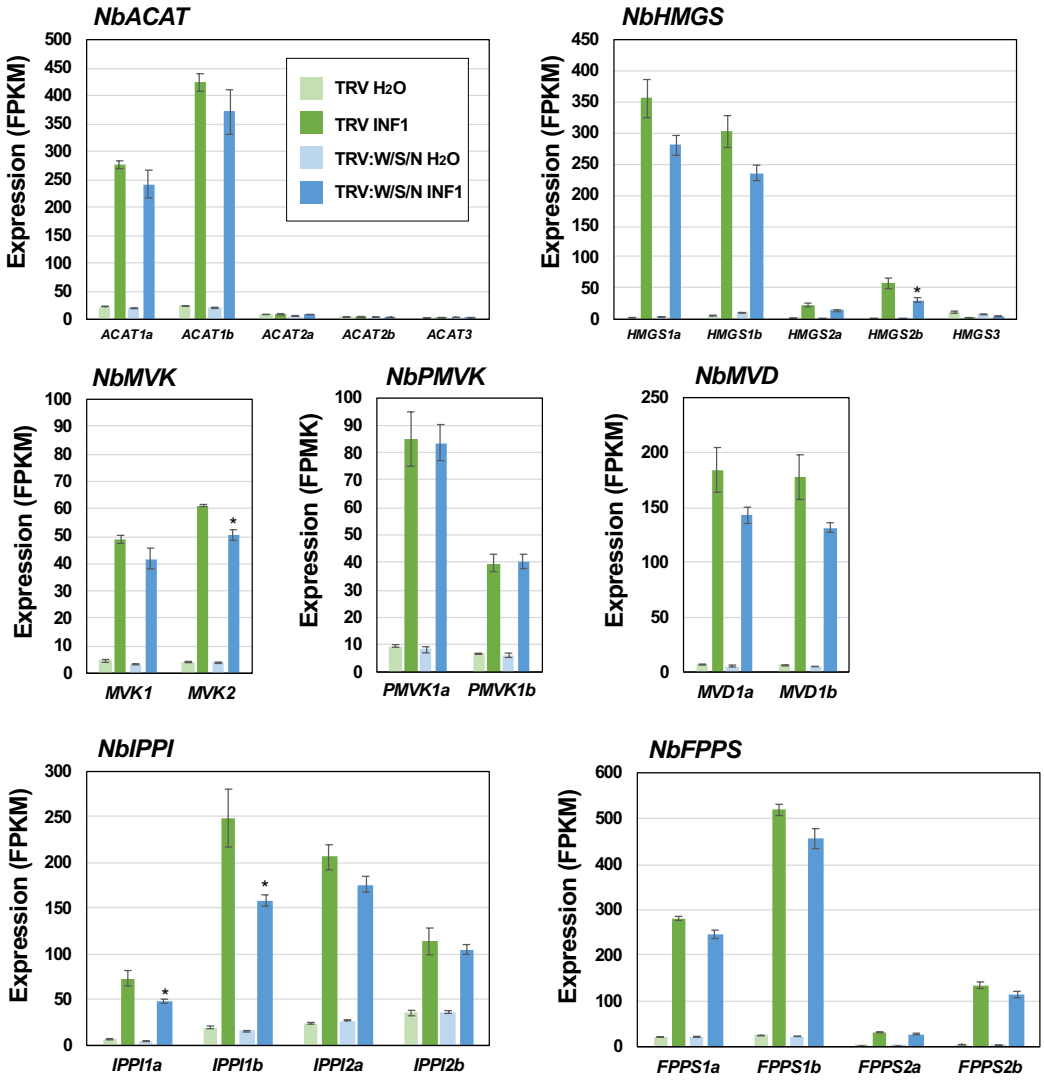


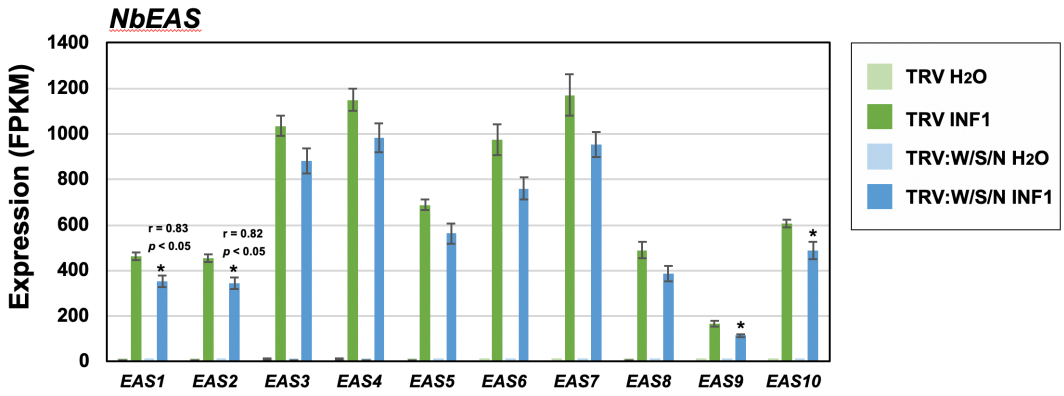
Fig. 4 Expression profiles of *Nicotiana benthamiana* genes encoding hydroxymethylglutaryl-CoA-reductase (HMGR) and 5-*epi*-aristolochene-1,3-dihydroxylase (EAH) in *NbWIPK/SIPK/NTF4*-silenced plants. Gene expression (FPKM value) was determined by RNA-seq analysis of TRV-inoculated control (TRV) or *NbWIPK/SIPK/NTF4*-silenced (TRV:W/S/N) *N. benthamiana* leaves treated with water (H_2O) or 150 nM INF1 for 24 h. Data are means ± SE (n = 3). Data marked with asterisks are significantly different from TRV-inoculated control as assessed by the two-tailed Student's *t* test: *P<0.05, **P<0.01. Results of Pearson correlation coefficient analysis are shown above the column in case that expression pattern of the gene showed significant positive correlation (r > 0.8, p < 0.05) with that of *NbWIPK/SIPK/NTF4*. Examples of PCC analysis (for *NbHMGR2* and *NbEAH5*) are shown in right graphs.



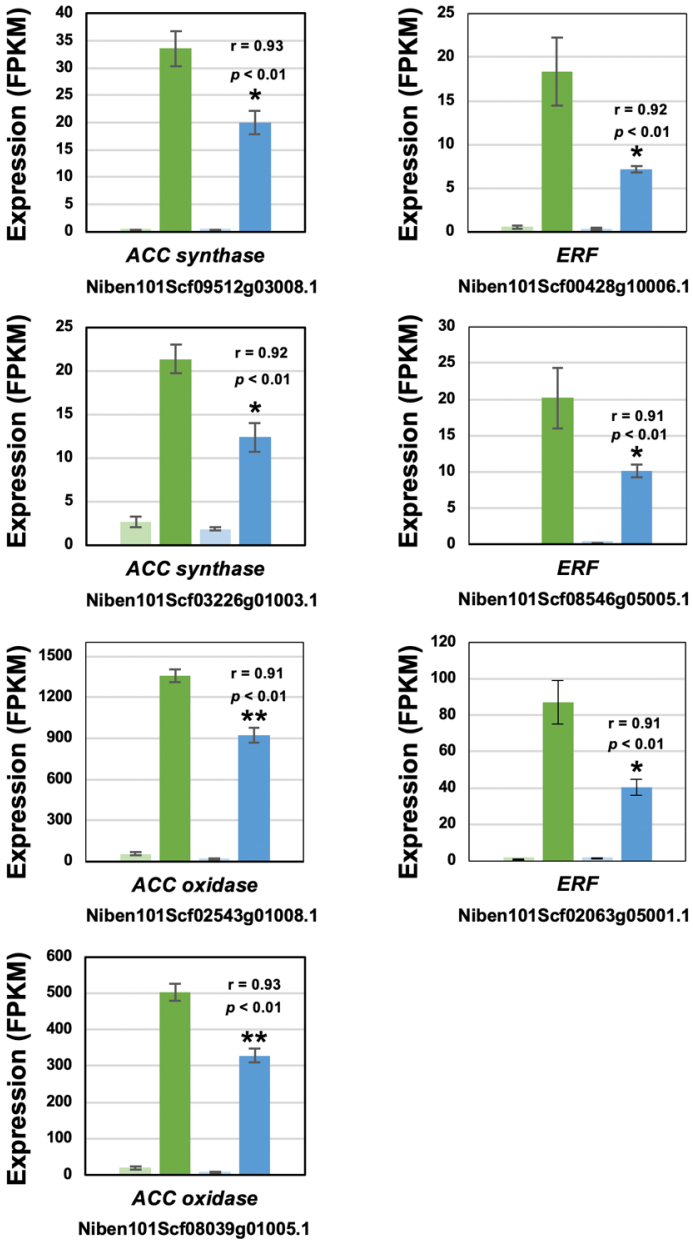
Supplementary Fig. S1 Biomass of *Phytophthora infestans* on *Nicotiana benthamiana* leaves. **a** Leaves of *N. benthamiana* were inoculated with zoospore suspension of *P. infestans* and ratio of transcript (mapped reads) from *P. infestans* and *N. benthamiana* was calculate at 24 and 48 h post inoculation (hpi) by RNA-seq analysis. Data are means ±SE (n = 3). Data marked with asterisks are significantly different from 0 h as assessed by the two-tailed Student's *t* test: **P<0.01. **b** Leaves of *N. benthamiana* were stained with lactophenol trypan blue to visualize dead plant cells and the hyphae of *P. infestans* 24 and 48 hpi. Bars = 20 µm.



Supplementary Fig. S2 Expression profiles of *Nicotiana benthamiana* genes encoding enzymes in mevalonate pathway and farnesyl pyrophosphate synthase. Gene expression (FPKM value) was determined by RNA-seq analysis of TRV-inoculated control (TRV) or *NbWIPK/SIPK/NTF4*-silenced (TRV:W/S/N) *N. benthamiana* leaves treated with water (H_2O) or 150 nM INF1 for 24 h. Data are means ±SE (n = 3). Data marked with asterisks are significantly different from TRV-inoculated control as assessed by the two-tailed Student's *t* test: *P<0.05. Results of Pearson correlation coefficient (PCC) analysis are shown above the columns where expression patterns showed significant positive correlation ($r > 0.8$, $p < 0.05$) with that of *NbWIPK/SIPK/NTF4*. ACAT, Acetoacetyl-CoA thiolase; HMGS, Hydroxymethylglutaryl-CoA-reductase; MVK, Mevalonate-5-kinase; PMVK, Phosphomevalonate kinase; MVD, Mevalonate-5-pyrophosphate decarboxylase; IPPI, Isopentenyl pyrophosphate isomerase; FPPS, Farnesyl pyrophosphate synthase.



Supplementary Fig. S3 Expression profiles of *Nicotiana benthamiana* 5-*epi*-aristolochene synthase (EAS) genes in *NbWIPK/SIPK/NTF4*-silenced plants. Gene expression (FPKM value) was determined by RNA-seq analysis of TRV-inoculated control (TRV) or *NbWIPK/SIPK/NTF4*-silenced (TRV:W/S/N) *N. benthamiana* leaves treated with water (H_2O) or 150 nM INF1 for 24 h. Data are means ±SE (n = 3). Data marked with asterisks are significantly different from TRV-inoculated control as assessed by the two-tailed Student's *t* test: *P<0.05. Results of Pearson correlation coefficient analysis are shown above the columns where expression patterns showed significant positive correlation ($r > 0.8$, $p < 0.05$) with that of *NbWIPK/SIPK/NTF4.*



Supplementary Fig. S4 Expression profiles of *Nicotiana benthamiana* 1-aminocyclopropane-1-carboxylate (ACC) synthases, ACC oxidases and ethylene-responsive transcription factor (ERF) in *NbWIPK/SIPK/NTF4*-silenced plants. Gene expression (FPKM value) was determined by RNA-seq analysis of TRV-inoculated control (TRV) or *NbWIPK/SIPK/NTF4*-silenced (TRV:W/S/N) *N. benthamiana* leaves treated with water (H_2O) or 150 nM INF1 for 24 h. Data are means ±SE (n = 3). Data marked with asterisks are significantly different from TRV-inoculated control as assessed by the two-tailed Student's *t* test: *P<0.05, **P<0.01. Results of Pearson correlation coefficient analysis are shown above the columns.

Supplementary Table S1 Predicted genes for enzymes in mevalonate pathway and farnesylpyrophosphate synthase from *Nicotiana benthamiana* genome sequence.

Gene Name	Gene ID
Acetyl-CoA thiolase	
ACAT1a	Niben101Scf04727g03006.1
ACAT1b	Niben101Scf01100g01006.1
ACAT2a	Niben101Scf06423g02006.1
ACAT2b	Niben101Scf01974g00019.1
ACAT3	Niben101Scf05433g00004.1
3-hydroxy-3-methylglutaryl-CoA synthase	
HMGS1a	Niben101Scf01111g01003.1
HMGS1b	Niben101Scf01729g01015.1
HMGS2a	Niben101Scf03321g01012.1
HMGS2b	Niben101Scf02361g01002.1
HMGS3	Niben101Scf10595g01007.1
3-hydroxy-3-methylglutaryl-CoA reductase	
HMGR1a	Niben101Scf13180g01003.1
HMGR1b	Niben101Scf09686g00013.1
HMGR2	Niben101Scf02203g05002.1
HMGR3a	Niben101Scf09883g01009.1
HMGR3b	Niben101Scf00163g01009.1
Mevalonate-5-kinase	
MVK1a	Niben101Scf25893g00005.1
MVK1b	Niben101Scf00370g03023.1
Phosphomevalonate kinase	
PMVK1a	Niben101Scf07030g04004.1
PMVK1b	Niben101Scf09628g00020.1
Mevalonate-5-pyrophosphate decarboxylase	
MVD1a	Niben101Scf03413g01011.1
MVD1b	Niben101Scf00173g05015.1
Isopentenyl pyrophosphate isomerase	
IPPI1a	Niben101Scf17839g02005.1
IPPI1b	Niben101Scf05848g05012.1
IPPI2a	Niben101Scf02499g03007.1
IPPI2b	Niben101Scf01514g04018.1
Farnesylpyrophosphate synthase	
FPPS1a	Niben101Scf04739g01006.1
FPPS1b	Niben101Scf00414g07005.1
FPPS2a	Niben101Scf04847g02011.1
FPPS2b	Niben101Scf04444g09012.1

Supplementary Table S2 Predicted genes for specific enzymes involved in the production of capsidiol.

Gene Name	Gene ID
5-*epi*-aristolochene synthase	
NbEAS1	Niben101Scf07725g01004.1
NbEAS2	Niben101Scf07725g00004.1
NbEAS3	Niben101Scf03993g06006.1
NbEAS4	Niben101Scf03993g05005.1
NbEAS5	Niben101Scf06245g01017.1
NbEAS6	Niben101Scf00700g00005.1
NbEAS7	Niben101Scf00712g02011.1
NbEAS8	Niben101Scf04362g07009.1
NbEAS9	Niben101Scf01683g03005.1
NbEAS10	Niben101Scf03400g02010.1
5-*epi*-aristolochene dihydroxylase	
NbEAH1	Niben101Scf00072g06001.1
NbEAH2	Niben101Scf04362g07011.1
NbEAH3	Niben101Scf00072g05003.1
NbEAH4	Niben101Scf00072g05004.1
NbEAH5	Niben101Scf00994g00001.1
NbEAH6	Niben101Scf04869g00002.1

Supplementary Table 3 List of *Nicotiana benthamiana* genes showing significant correlation with expression profiles of *NbWIPK/SIPK/NTF4* in leaves.

Gene ID	TRV_H2O [FPKM]	TRV_INF1 [FPKM]	TRV:W/S/N_H2O [FPKM]	TRV:W/S/N_INF1 [FPKM]	Log FC (INF1/H2O)	P value (INF1/H2O)	PCC analysis with NbWIPK/SIPK/NTF4 (*r* value)	PCC analysis with NbWIPK (*r* value)	PCC analysis with NbSIPK (*r* value)	PCC analysis with NbNTF4 (*r* value)	Annotation
Niben101Scf06162g01008.1	0.00	11.87	0.16	3.59	>10	3.5.E-04	0.996	0.976	0.970	0.974	Unknown protein
Niben101Scf15809g00002.1	0.00	12.96	0.00	5.85	>10	1.1.E-04	0.955	0.925	0.946	0.959	Sodium transporter HKT1-like [Nicotiana sylvestris]
Niben101Scf00803g01016.1	0.00	58.50	0.12	19.59	>10	3.6.E-03	0.932	0.917	0.901	0.901	Hypothetical protein A4A49_55490 [Nicotiana attenuata]
Niben101Ctg16292g00001.1	0.00	20.69	0.00	11.37	>10	7.3.E-05	0.914	0.892	0.896	0.899	E3 ubiquitin-protein ligase rha2b [Nicotiana attenuata]
Niben101Scf01313g02004.1	0.00	20.69	0.00	11.37	>10	7.3.E-05	0.914	0.892	0.896	0.899	E3 ubiquitin-protein ligase rha2b [Nicotiana attenuata]
Niben101Scf08546g05005.1	0.00	20.21	0.16	10.11	>10	8.3.E-03	0.911	0.959	0.775	0.783	Ethylene-responsive transcription factor ERF106 [Nicotiana sylvestris]
Niben101Scf01313g01005.1	0.00	72.81	0.00	37.57	>10	2.1.E-04	0.907	0.909	0.849	0.856	E3 ubiquitin-protein ligase Os06g0535400-like [Nicotiana sylvestris]
Niben101Scf09260g03026.1	0.00	10.19	0.00	3.17	>10	1.1.E-02	0.900	0.928	0.795	0.816	U-box domain-containing protein 21-like [Nicotiana attenuata]
Niben101Scf04800g02001.1	0.05	34.48	0.00	13.23	9.54	5.7.E-05	0.947	0.898	0.974	0.967	Elongation of fatty acids protein 3-like [Nicotiana sylvestris]
Niben101Scf03226g03006.1	0.28	117.89	0.00	61.19	8.73	1.1.E-08	0.902	0.860	0.918	0.919	Uncharacterized protein LOC104216072 [Nicotiana sylvestris]
Niben101Scf08855g02005.1	0.10	41.62	0.02	13.45	8.72	7.9.E-04	0.973	0.963	0.932	0.937	U-box domain-containing protein 21-like [Nicotiana sylvestris]
Niben101Scf11334g01003.1	0.21	80.36	0.08	48.17	8.57	7.6.E-05	0.916	0.884	0.911	0.922	Uncharacterized protein At1g28695-like [Nicotiana attenuata]
Niben101Scf00163g09011.1	0.29	105.72	0.12	63.18	8.49	5.8.E-06	0.901	0.851	0.932	0.931	Uncharacterized protein At1g28695-like [Nicotiana attenuata]
Niben101Scf02063g05001.1	0.51	86.99	1.62	40.19	7.43	1.9.E-03	0.906	0.887	0.886	0.884	Ethylene-responsive transcription factor 1B-like [Nicotiana attenuata]
Niben101Scf09512g03008.1	0.20	33.58	0.36	20.06	7.39	4.6.E-04	0.927	0.944	0.841	0.854	1-aminocyclopropane-1-carboxylate synthase-like [Nicotiana tabacum]
Niben101Scf02349g03001.1	2.50	317.02	2.38	164.20	6.99	1.2.E-06	0.929	0.892	0.934	0.939	Suberization-associated anionic peroxidase-like [Nicotiana sylvestris]
Niben101Scf00240g01002.1	0.50	54.19	0.21	26.21	6.75	4.3.E-04	0.975	0.973	0.919	0.925	RING-H2 finger protein ATL60-like [Nicotiana sylvestris]
Niben101Scf02437g01001.1	0.14	15.20	0.06	11.37	6.75	3.1.E-04	0.909	0.935	0.811	0.813	VQ motif-containing protein 22-like [Nicotiana tabacum]
Niben101Scf07440g00009.1	0.38	40.28	0.35	26.66	6.72	1.0.E-04	0.945	0.923	0.928	0.921	GEM-like protein 6 [Nicotiana tabacum]
Niben101Scf14778g00021.1	1.23	124.86	0.43	46.98	6.67	8.7.E-05	0.913	0.849	0.966	0.965	E3 ubiquitin-protein ligase ATL31-like [Nicotiana tabacum]
Niben101Scf00994g00001.1	2.40	213.87	0.40	144.48	6.48	3.8.E-06	0.959	0.929	0.952	0.947	NbEAH5 [Nicotiana benthamiana]
Niben101Scf01402g01007.1	0.29	24.05	0.15	7.59	6.36	1.4.E-03	0.939	0.907	0.937	0.934	Galacturonosyltransferase-like 10 [Nicotiana attenuata]
Niben101Scf01398g00006.1	2.20	141.78	1.64	85.24	6.01	1.5.E-07	0.912	0.868	0.932	0.927	Putative late blight resistance protein homolog R1B-16 [Nicotiana sylvestris]
Niben101Scf02636g07002.1	0.81	50.85	0.00	18.70	5.97	1.6.E-02	0.909	0.943	0.797	0.799	Hypothetical protein A4A49_07303 [Nicotiana attenuata]
Niben101Scf08624g00006.1	0.54	32.26	0.03	8.77	5.89	1.0.E-05	0.962	0.913	0.985	0.990	Mitogen-activated protein kinase kinase kinase NPK1-like [Nicotiana attenuata]
Niben101Scf01739g08010.1	1.35	77.20	0.14	37.83	5.84	3.8.E-04	0.946	0.924	0.926	0.931	VQ motif-containing protein 22 [Nicotiana attenuata]
Niben101Scf00522g01017.1	1.37	73.69	0.64	50.25	5.74	9.6.E-05	0.993	0.995	0.930	0.935	Transcription factor MYB57-like [Nicotiana sylvestris]
Niben101Scf05453g00018.1	0.71	35.75	0.21	10.34	5.65	1.3.E-02	0.925	0.942	0.842	0.844	BON1-associated protein 2-like [Nicotiana sylvestris]
Niben101Scf01569g02006.1	3.80	189.35	1.21	126.81	5.64	1.2.E-04	0.921	0.889	0.922	0.905	Non-specific lipid-transfer protein A-like [Nicotiana sylvestris]
Niben101Scf01569g01001.1	2.38	112.79	0.56	75.90	5.57	9.5.E-05	0.966	0.950	0.938	0.930	Non-specific lipid-transfer protein A-like [Nicotiana sylvestris]
Niben101Scf04973g02010.1	2.06	94.37	0.25	33.85	5.52	6.0.E-03	0.952	0.971	0.864	0.867	Uncharacterized protein LOC109241892 [Nicotiana attenuata]
Niben101Scf04988g02019.1	3.08	128.83	1.28	50.31	5.39	1.8.E-04	0.918	0.865	0.951	0.949	BTB/POZ domain-containing protein At5g41330-like [Nicotiana tabacum]
Niben101Scf02381g04006.1	0.62	25.04	0.31	15.34	5.34	1.5.E-04	0.913	0.890	0.896	0.903	Caffeoylshikimate esterase-like [Nicotiana attenuata]
Niben101Scf06280g01016.1	1.60	55.55	0.48	18.59	5.12	2.6.E-03	0.967	0.966	0.910	0.915	Probable galacturonosyltransferase-like 10 [Nicotiana attenuata]
Niben101Scf06424g00001.1	1.68	57.48	1.41	28.79	5.10	1.7.E-04	0.935	0.883	0.967	0.961	GEM-like protein 4 [Nicotiana sylvestris]
Niben101Scf04003g01002.1	0.81	27.26	0.24	8.11	5.08	3.7.E-03	0.973	0.973	0.914	0.916	Mitogen-activated protein kinase kinase kinase NPK1-like [Nicotiana attenuata]
Niben101Scf09445g04018.1	0.72	24.12	0.36	17.40	5.06	6.2.E-04	0.937	0.978	0.814	0.816	Ankyrin repeat-containing protein At3g12360-like [Nicotiana tabacum]
Niben101Scf00428g10006.1	0.56	18.37	0.30	7.18	5.04	1.1.E-02	0.923	0.943	0.837	0.829	Ethylene-responsive transcription factor (ERF) 1A-like [Nicotiana attenuata]
Niben101Scf05200g00003.1	0.33	10.31	0.09	2.92	4.95	1.5.E-03	0.938	0.896	0.954	0.947	Mitogen-activated protein kinase kinase kinase NPK1-like [Nicotiana tomentosiformis]
Niben101Scf00317g04009.1	0.44	13.32	0.28	4.74	4.92	3.2.E-06	0.956	0.915	0.967	0.968	Metalloendoproteinase 4-MMP-like [Nicotiana attenuata]
Niben101Scf00927g08010.1	0.98	27.36	0.22	12.96	4.80	5.0.E-04	0.925	0.906	0.901	0.907	U-box domain-containing protein CMPG1a [Nicotiana benthamiana]
Niben101Scf08039g01005.1	19.89	503.39	5.37	328.07	4.66	3.3.E-05	0.931	0.897	0.933	0.930	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) [Nicotiana benthamiana]
Niben101Scf03877g00018.1	2.05	51.62	0.44	31.10	4.66	1.2.E-05	0.960	0.931	0.953	0.948	F-box protein CPR30-like [Nicotiana sylvestris]
Niben101Scf02543g01008.1	55.46	1357.53	15.31	920.72	4.61	1.3.E-05	0.910	0.865	0.931	0.930	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) [Nicotiana benthamiana]
Niben101Scf00662g04008.1	2.73	66.16	0.33	14.53	4.60	7.6.E-03	0.974	0.976	0.913	0.911	Zinc finger protein ZAT10-like [Nicotiana sylvestris]
Niben101Scf06517g00007.1	0.73	17.09	0.00	9.98	4.54	5.4.E-05	0.905	0.872	0.909	0.900	MADS-box transcription factor ANR1 [Vitis vinifera]
Niben101Scf02460g01004.1	1.24	27.05	0.36	12.14	4.45	1.1.E-04	0.927	0.889	0.935	0.938	Avr9/Cf-9 induced kinase 1 [Nicotiana tabacum]
Niben101Scf01998g01003.1	1.08	23.30	1.17	14.28	4.44	2.6.E-04	0.901	0.850	0.935	0.932	Uncharacterized protein LOC104238174 [Nicotiana sylvestris]
Niben101Scf02167g00009.1	3.35	64.29	1.91	33.37	4.26	2.9.E-06	0.916	0.852	0.969	0.970	Phospholipid-transporting ATPase 1 [Capsicum annuum]
Niben101Scf05060g07008.1	8.90	167.53	4.38	79.03	4.23	1.1.E-02	0.902	0.939	0.788	0.789	Probable calcium-binding protein CML45 [Nicotiana tomentosiformis]
Niben101Scf17237g00001.1	5.98	106.57	1.12	28.33	4.15	1.6.E-04	0.960	0.912	0.984	0.984	Zinc finger protein ZAT10-like [Nicotiana attenuata]
Niben101Scf28763g00006.1	2.78	49.33	0.28	11.56	4.15	1.2.E-02	0.921	0.905	0.895	0.888	Zinc finger protein ZAT10-like [Nicotiana sylvestris]
Niben101Scf01413g02004.1	0.92	16.06	0.93	5.88	4.13	2.4.E-04	0.957	0.928	0.947	0.947	Internal alternative NAD(P)H-ubiquinone oxidoreductase A2-like [Nicotiana tabacum]
Niben101Scf06739g05004.1	1.28	22.38	0.31	7.86	4.13	8.7.E-04	0.922	0.885	0.930	0.929	Serine/threonine-protein kinase At5g01020-like [Nicotiana tabacum]
Niben101Scf13180g01003.1	13.17	184.25	6.84	99.99	3.81	1.8.E-04	0.960	0.937	0.938	0.947	NbHMGR1a [Nicotiana benthamiana]
Niben101Scf02838g07002.1	6.29	87.18	4.43	63.51	3.79	1.4.E-04	0.905	0.926	0.813	0.828	Cucumber peeling cupredoxin-like [Nicotiana attenuata]
Niben101Scf01649g04003.1	2.42	32.14	2.11	9.83	3.73	1.9.E-02	0.930	0.959	0.826	0.829	Uncharacterized protein LOC109242456 [Nicotiana attenuata]
Niben101Scf10316g03002.1	5.14	66.06	6.95	48.63	3.68	1.5.E-06	0.927	0.892	0.929	0.936	Probable glutathione S-transferase [Nicotiana tomentosiformis]
Niben101Scf06017g02002.1	1.77	22.60	0.28	10.58	3.68	3.1.E-05	0.988	0.952	0.989	0.991	Myb-related protein Myb4-like [Nicotiana tabacum]
Niben101Scf10205g00006.1	1.96	25.00	1.31	13.03	3.68	1.2.E-05	0.902	0.833	0.964	0.963	Probable glutathione S-transferase [Nicotiana sylvestris]
Niben101Scf01956g10006.1	1.09	11.95	1.17	9.66	3.46	7.9.E-07	0.924	0.851	0.991	0.990	Uncharacterized protein LOC109210411 [Nicotiana attenuata]
Niben101Scf02807g10009.1	6.76	74.18	5.65	49.79	3.45	1.8.E-04	0.976	0.977	0.917	0.918	VQ motif-containing protein 22-like [Nicotiana tabacum]
Niben101Scf05883g02007.1	14.56	158.89	5.96	102.18	3.45	6.2.E-04	0.910	0.893	0.883	0.891	Polyol transporter 5-like [Nicotiana sylvestris]
Niben101Scf01013g01003.1	1.30	13.83	0.35	5.87	3.41	2.1.E-03	0.985	0.991	0.917	0.916	Myb-related protein Myb4-like [Nicotiana sylvestris]
Niben101Scf00080g05006.1	15.41	161.98	8.06	72.22	3.39	1.2.E-02	0.918	0.959	0.791	0.799	Zinc finger protein PIF1 [Nicotiana benthamiana]
Niben101Scf00383g04025.1	2.06	19.35	2.00	14.55	3.23	5.3.E-06	0.909	0.891	0.883	0.892	Late embryogenesis abundant protein-like [Nicotiana sylvestris]
Niben101Scf03908g02006.1	14.25	133.61	5.29	88.14	3.23	2.5.E-05	0.969	0.929	0.980	0.971	Protein BPS1, chloroplastic-like [Nicotiana sylvestris]
Niben101Scf06162g01006.1	2.51	21.48	0.33	12.91	3.10	9.6.E-04	0.973	0.986	0.893	0.897	O-fucosyltransferase 20-like [Solanum lycopersicum]
Niben101Scf01589g00002.1	2.90	24.19	0.69	14.93	3.06	2.1.E-03	0.915	0.929	0.837	0.841	EGF domain-specific O-linked N-acetylglucosamine transferase [Nicotiana sylvestris]
Niben101Scf03226g01003.1	2.64	21.36	1.87	12.37	3.01	4.9.E-04	0.922	0.916	0.872	0.894	1-aminocyclopropane-1-carboxylate synthase (ACS)-like [Nicotiana sylvestris]
Niben101Scf00586g00007.1	6.88	55.29	5.67	36.96	3.01	6.0.E-06	0.924	0.867	0.966	0.961	Aldo-keto reductase family 4 member C10-like [Nicotiana tabacum]

N. benthamiana genes showed significant increased expression by INF1 treatment at 24 h (LogFC > 3) are selected for Pearson correlation coefficient (PCC) analysis with expression profiles of *NbWIPK/SIPK/NTF4*.

Genes with expression profile closely correlation with *NbWIPK/SIPK/NTF4* (r value > 9.0) were listed in this table.