Nucleoporin 75 Is Involved in the Ethylene-Mediated Production of Phytoalexin for the Resistance of *Nicotiana benthamiana* to *Phytophthora infestans*

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Mature *Nicotiana benthamiana* shows stable resistance to the oomycete pathogen *Phytophthora infestans*. Induction of phytoalexin (capsidiol) production is essential for the resistance, which is upregulated via a mitogen-activated protein kinase (MAPK) cascade (NbMEK2-WIPK/SIPK) followed by ethylene signaling. In this study, *NbNup75* (encodes a nuclear pore protein Nucleoporin75) was identified as an essential gene for resistance of *N. benthamiana* to *P. infestans*. In *NbNup75*-silenced plants, initial events of elicitor-induced responses such as phosphorylation of MAPK and expression of defense-related genes were not affected, whereas induction of later defense responses such as capsidiol production and cell death induction was suppressed or delayed. Ethylene production induced by either INF1 or NbMEK2 was reduced in *NbNup75*-silenced plants, whereas the expression of *NbEAS* (a gene for capsidiol biosynthesis) induced by ethylene was not affected, indicating that Nup75 is required for the induction of ethylene production but not for ethylene signaling. Given that nuclear accumulation of polyA RNA was increased in *NbNup75*-silenced plants, efficient export of mRNA from nuclei via nuclear pores would be important for the timely upregulation of defense responses. Collectively, Nup75 is involved in the induction of a later stage of defense responses, including the ethylene-mediated production of phytoalexin for the resistance of *N. benthamiana* to *P. infestans*.

The oomycete pathogen *Phytophthora infestans* is the causal agent of potato late blight, one of the most destructive and economically significant plant diseases. *P. infestans* is a historically significant pathogen, being the cause of Irish potato famine in the 1840s, and, even now, the total cost of control efforts and yield losses caused by *P. infestans* is estimated at more than \$3 billion per year (Fry 2008). Although *P. infestans* is an economically important pathogen, genes required for plant resistance against *P. infestans* are largely unknown except for resistance (*R*) genes for race-specific resistance, perhaps

because potato, the natural host of *P. infestans*, is not an ideal plant material for molecular biology. Recently, *Nicotiana benthamiana*, a wild *Nicotiana* sp. endemic to Australia, has been widely applied as a Solanaceae model plant for functional analysis of genes by manipulating expression of target genes using *Agrobacterium*-mediated transient gene expression or virus-induced gene silencing (VIGS) (Goodin et al. 2008; Ratcliff et al. 2001). We previously reported that mature *N. benthamiana* showed resistance to *P. infestans*, whereas rather young plants were susceptible to all of tested *P. infestans* isolates, indicating that *N. benthamiana* has an age-dependent resistance against *P. infestans* (Shibata et al. 2011). Therefore, mature *N. benthamiana* can be employed as a model host plant to investigate plant genes required for the resistance to *P. infestans*.

We have identified several genes involved in the resistance of mature *N. benthamiana* to *P. infestans*. Analysis with VIGS revealed that *NbEIN2* (ethylene insensitive 2), a gene for ethylene signaling, and *NbEAS* (5-*epi*-aristrochen synthase) and *NbEAH* (5-*epi*-aristrochen dihydroxylase), genes for phytoalexin biosynthesis, are required for the resistance of *N. benthamiana* against *P. infestans* (Shibata et al. 2010). Expression of *NbEAS* and *NbEAH* are induced by the ethylene treatment and suppressed by silencing of *NbEIN2*, indicating that the production of phytoalexin is regulated by ethylene in *N. benthamiana* (Shibata et al. 2010). Mechanisms for the induction of phytoalexin production are diverse among both plant species and the metabolic pathways involved. There are several reports indicating the important role of ethylene for the induction of phytoalexin production in different plant species (Chung et al. 2001; Ishigaki et al. 2004; Nakazato et al. 2000; Shibata et al. 2010). In rice, production of sakuranetin but not momilactone A was induced by ethylene (Nakazato et al. 2000). In *Arabidopsis*, production of an indole alkaloid phytoalexin, camalexin, is dependent on a mitogen-activated protein kinase (MAPK) cascade (MKK4/5-MPK3/6) (Ren et al. 2008) but independent from the ethylene signaling pathway. *Arabidopsis ein2* mutant can produce camalexin in response to necrotrophic fungal pathogens or expression of the constitutive active form of MAPK kinase (Ren et al. 2008; van Wees et al. 2003).

There are two crucial steps for production of phytoalexins mediated by ethylene. The first step is the induction of ethylene production in response to pathogen attack. Ethylene biosynthesis starts from the conversion of S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by ACC synthase (ACS), followed by the oxidative cleavage of ACC by ACC oxidase (ACO) (Wang et al. 2002;

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Yang and Hoffman 1984; Zarembinski and Theologis 1994). Activation of ACS is considered as the rate-limiting step for the production of ethylene. ACS is encoded by a gene family in all plant species (e.g., 9 and at least 12 ACS genes in *Arabidopsis* and tomato, respectively). Expression of some members of plant ACS genes are highly responsive to environmental stimuli such as wounding and attack of fungal pathogens (Broekaert et al. 2006; Tsuchisaka and Theologis 2004; van Loon et al. 2006). Recent reports indicate that the expression of *Arabidopsis* ACS genes, *AtACS2* and *AtACS6*, is directly controlled by a transcription factor, *AtWRKY33*, phosphorylated via an MAPK (MKK4/5-MPK3/6) cascade (Li et al. 2012).

The second step is the activation of ethylene signaling leading to the induction of enzymes involved in phytoalexin production. Perception of ethylene by endoplasmic reticulum receptors (ETR1/2, ERS1/2, and EIN4 in *Arabidopsis*) leads to the activation of ERF transcription factors to regulate the expression of target genes (Broekaert et al. 2006). Although the expression of *NbEAS* and *NbEAH* is regulated via ethylene signaling (Shibata et al. 2010), it remains to be clarified how the transcriptional activation of these genes is regulated in *N. benthamiana*.

In this study, VIGS-based screening was employed to isolate genes required for the resistance of *N. benthamiana* against *P. infestans*. One of the isolated genes encoded Nucleoporin 75 (Nup75), a component of the nuclear pore complex (NPC). Nucleoporins mediate the transport of macromolecules, such as proteins and RNAs, between cytoplasm and nucleoplasm across the nuclear envelope. Genes for nucleoporins from *Lotus japonicus*, *Nup85* (Nup75) and *Seh1*, were previously identified as essential genes for the establishment of symbiosis with arbuscular mycorrhizal fungi and rhizobial bacteria (Groth et al. 2010; Saito et al. 2007). *Arabidopsis mos7/nup88* mutants showed reduced basal and *R*-gene dependent resistance against bacterial pathogens (Cheng et al. 2009). More recently, *Arabidopsis* *Seh1* but not *Nup85* (Nup75) has been reported to play a role in resistance against *Pseudomonas syringae* (Wiermer et al. 2012). To reveal the function of Nup75 in the resistance of *N. benthamiana* against *Phytophthora infestans*, the effect of *NbNup75*-silencing on the induction of various resistance reactions was investigated. We particularly focused on the phytoalexin production mediated by MAPK and ethylene to reveal the processes of signal transduction affected by the silencing of *NbNup75*.

RESULTS

Nup75, a component of the NPC, is required for the resistance of *N. benthamiana* to *P. infestans*.

To identify genes required for the resistance of *N. benthamiana* to *P. infestans*, we randomly cloned cDNA fragments prepared from *N. benthamiana* leaves treated with INF1, a secretory elicitor protein of *P. infestans* (Kamoun et al. 1997), in the vector pTV00 for VIGS (Matsukawa et al. 2013; Ratcliff et al. 2001). Approximately 2,000 *N. benthamiana* plants with random silencing of genes were generated and inoculated with *P. infestans* isolate 08YD1. In all, 63 plants showed development of disease symptoms within 10 days after the inoculation with *P. infestans*. Twenty-six unique genes were isolated from 63 plant lines as candidate genes required for the resistance to *P. infestans*. In this study, we focused on the analysis of candidate line A2-266. Further analyses of other lines are in progress and will be reported elsewhere (Matsukawa et al. 2013).

In primary screening for *N. benthamiana* lines showing reduced resistance against *P. infestans*, A2-266 displayed signifi-

cant development of disease symptoms (Fig. 1). The vector used for the induction of VIGS for line A2-266 was isolated from *Agrobacterium tumefaciens* and inserted cDNA fragments were sequenced. The silencing vector for A2-266 contained a 321-bp cDNA fragment encoding Nup75, a component of the NPC (Tamura et al. 2010). Analysis of the draft genome sequence of *N. benthamiana* (ver. 0.4.4; Sol Genomics Network) (Bombarely et al. 2012) and RNAseq data that we performed identified two copies of *NbNup75*, designated *NbNup75a* and *NbNup75b* (Supplementary Fig. S1; Supplementary Table S1). Because *N. benthamiana* has an allopolyploid genome (Goodin et al. 2008), we often find highly conserved, double copies of genes in the genome of *N. benthamiana* (Matsukawa et al. 2013). Two copies of *NbNup75* could have derived from different ancestral *Nicotiana* spp. and it is very likely that they are functionally redundant. Because *NbNup75a* and *75b* showed 97% identity in nucleic acid sequence, expression of both *NbNup75* genes was suppressed in *NbNup75*-silenced plants (Supplementary Fig. S2). To confirm the importance of *NbNup75* in disease resistance of *N. benthamiana* to *P. infestans*, we constructed new VIGS vectors for effective silencing of *NbNup75*. Compared with the VIGS vector for A2-266 line (*NbNup75-1*), newly constructed VIGS vectors *NbNup75-2* and *NbNup75-3* can induce more effective gene silencing, and plants made with new vectors showed more severe disease symptoms compared with the original A2-266 line. Because the cDNA fragment used for the production of both *NbNup75-2*- and *NbNup75-3*-silenced plants did not show any significant identity with other genes in the *N. benthamiana* draft genome, it was indicated that specific target genes were suppressed in *NbNup75-2*- and *NbNup75-3*-silenced plants. *NbNup75-3*-silenced *N. benthamiana* were used as *NbNup75*-silenced plants in the following analysis.

Silencing of *NbNup75* did not alter plant development and did not affect nuclei morphology.

Recently, 30 *Arabidopsis* nucleoporins were identified (Tamura et al. 2010). Analysis of RNAseq data of *N. benthamiana* mapped on a draft genome sequence of *N. benthamiana* (ver. 0.4.4) identified 28 *N. benthamiana* nucleoporins (Supplementary Fig. S3A). Silencing vectors for *NbSeh1* and *NbNup88* were constructed to compare the role of these nucleoporins with that of *NbNup75* in resistance of *N. benthamiana* against *P. infestans*. Gene-specific fragments of *NbNup88* and *NbSeh1* were cloned into the pTV00 vector. It was confirmed that the expression of target genes was suppressed in VIGS-induced plants. The *NbNup75*- and *NbSeh1*-silenced plants showed almost normal growth, whereas *NbNup88*-silenced plants showed a semidwarf phenotype with shrunken leaves. Confocal microscopy of 4',6-diamidino-2-phenylindole-stained nuclei revealed that the nuclear structure of *NbNup75*- and *NbSeh1*-silenced plants was almost normal, whereas irregular shapes of nuclei were often observed in *NbNup88*-silenced plants. Therefore, fundamental defect of nuclear formation is likely the cause of growth defect in *NbNup88*-silenced plants. In contrast, *NbNup75* and *NbSeh1* are not essential for the normal growth of *N. benthamiana*, although previous analysis of the yeast Nup107-160 complex indicated that Nup75 and *Seh1* are structural nucleoporins that form the outer rings of the NPC (Grossman et al. 2012).

NbNup75, *NbNup88*, and *NbSeh1* are required for resistance of *N. benthamiana* to *P. infestans*.

NbNup75-, *NbNup88*-, and *NbSeh1*-silenced *N. benthamiana* plants were inoculated with *P. infestans*. Within 7 days after the inoculation of *P. infestans*, there was no significant development of disease symptoms for control plants, whereas

NbNup75-, *NbNup88*-, and *NbSeh1*-silenced plants showed disease symptoms on the inoculated leaves (Fig. 2A). Reduced resistance of *NbNup*-silenced plants was scored by visible development of disease symptoms (Fig. 2B). At 4 days after the inoculation, increased development of disease symptoms was observed for *NbNup75*-, *NbNup88*-, or *NbSeh1*-silenced plants. Increased biomass of *P. infestans* in *NbNup75*-, *NbNup88*-, or *NbSeh1*-silenced plants compared with control plants further confirmed that *NbNup75*, *NbNup88*, and *NbSeh1* are required for resistance of *N. benthamiana* to *P. infestans* (Fig. 2C). Ten days after the inoculation, hyphal extension in the plant mesophyll cell layer, emergence of hypha from stomatal openings, and production of zoospores were observed in *NbNup75*-, *NbNup88*-, or *NbSeh1*-silenced plants (Supplementary Fig. S4).

***NbNup75*, *NbNup88*, and *NbSeh1*-silenced *N. benthamiana* has compromised reactive oxygen species production induced by treatment with INF1 elicitor.**

Generation of reactive oxygen species (ROS) is a conserved feature of plant defense responses and ROS are considered to be signaling molecules for the induction of plant immunity (Doke 1983; Torres and Dangl 2005). Leaves of *NbNup75*-, *NbNup88*-, or *NbSeh1*-silenced or control *N. benthamiana* plants were treated with 150 nM INF1, and production of ROS (O_2^-) was measured as chemiluminescence using the luminol derivative L-012 at 12 h after treatment. *NbNup75*-, *NbNup88*-, and *NbSeh1*-silenced plants showed significant reduction of ROS production compared with the control plants (Fig. 3A and B).

Fig. 1. *Nicotiana benthamiana* plants with random gene silencing (approximately 2,000 individual plants) were inoculated with *Phytophthora infestans*. A candidate line A2-266 showed reduced resistance to *P. infestans*. *Tobacco rattle virus* (TRV)- and TRV:EAS-infected plants were used as resistant and susceptible control plants, respectively.

Fig. 2. *NbNup75*, *NbNup88*, and *NbSeh1* are essential for the resistance of *Nicotiana benthamiana* to *Phytophthora infestans*. **A**, Leaves of *NbNup75*-, *NbNup88*-, and *NbSeh1*-silenced or control *N. benthamiana* plants were inoculated with *P. infestans*. Photographs were taken 9 days after inoculation with *P. infestans*. **B**, Top: Appearance of disease symptoms on leaves. Based on severity, disease symptoms on leaves were categorized into five classes: 0 = no visible symptom, 1 = small wilted spots on inoculated area, 2 = wilting of whole inoculated area, 3 = browning of inoculated area, and 4 = development of disease symptom over central leaf vein. Bottom: Plot showing percentage of *N. benthamiana* leaves with disease symptom severities represented in the five classes as shown in upper panels for leaves of control and *NbNup75*-, *NbNup88*-, or *NbSeh1*-silenced plants inoculated with *P. infestans* from 2 to 5 days postinoculation (dpi). At least 10 inoculated leaves from each silenced plant were scored. **C**, Biomasses of *P. infestans* in *N. benthamiana* leaves were determined by quantitative reverse-transcriptase polymerase chain reaction 2 days after inoculation as relative expressions of the *PiActA* gene to that of *N. benthamiana* *NbEF-1α*. Data are means $\pm$ standard error ($n = 3$). Data marked with asterisks are significantly different from control (*Tobacco rattle virus* [TRV]) as assessed by the two-tailed Student's *t* test: * and ** indicate $P < 0.05$ and 0.01 , respectively. Results shown are representative of at least three separate experiments.

***NbNup75* and *NbSeh1* are essential for the accumulation of phytoalexin induced by the INF1 elicitor.**

Capsidiol is a major sesquiterpenoid phytoalexin produced by *Nicotiana* and *Capsicum* spp. (Bailey et al. 1975). Biosynthesis of capsidiol involves the cyclization of farnesyl pyrophosphate (FPP) to the sesquiterpenoid hydrocarbon 5-*epi*-aristolochene catalyzed by 5-*epi*-aristolochene synthase (EAS) (Facchini and Chappell 1992) and two subsequent hydroxylation steps by 5-*epi*-aristolochene dihydroxylase (EAH) (Ralston et al. 2001) (Fig. 4A). To investigate the role of nucleoporins in phytoalexin biosynthesis, the production of capsidiol was quantified in gene-silenced *N. benthamiana*. At 12 h after treatment with 150 nM INF1, capsidiol was extracted from *Tobacco rattle virus* (TRV)-infected, *NbEAS*-silenced, and *NbNup*-silenced plants and the amount of capsidiol produced was quantified by high-perfor-

mance liquid chromatography. Production of capsidiol by *NbNup88*-silenced *N. benthamiana* was comparable with that of the TRV-infected plant. In contrast, production of capsidiol in *NbNup75*- and *NbSeh1*-silenced plants was significantly reduced (Fig. 3C). The resistance of *NbNup75*-silenced plants but not *NbNup88*-silenced plants against *P. infestans* was recovered by the pretreatment of capsidiol, indicating that the compromised production of capsidiol is a major cause of reduced resistance of *NbNup75*-silenced plants (Supplementary Fig. S5). Given that INF1-induced expression of *NbEAS* was repressed by gene silencing of either *NbNup75* or *NbSeh1*, impaired production of capsidiol in *NbNup75*- and *NbSeh1*-silenced plants appeared to be a consequence of the reduced expression of genes for phytoalexin biosynthesis (Fig. 3D).

***NbNup75* is required for the INF1-induced production of ethylene but not for the response to ethylene.**

Previously, we have reported that the expression of the *NbEAS* gene was upregulated by ethylene treatment (Shibata et al. 2010). Expression analysis of genes for enzymes in the mevalonate pathway indicated that expression of some tested

genes was also induced by ethylene treatment (Fig. 4A). Consistently, *NbEIN2*-silenced plants showed susceptibility to *P. infestans* (Shibata et al. 2010), and the accumulation of capsidiol in *EIN2*-silenced plants was reduced (Supplementary Fig. S6). To investigate the effect of *NbNup75* silencing on ethylene response and production, we first investigated the expression of *NbEAS* in *N. benthamiana* exposed to ethylene in control, *NbEIN2*-, and *NbNup75*-silenced plants. At 6 h after the treatment with 100 ppm ethylene, the expression of *NbEAS* was observed in both the control and *NbNup75*-silenced plants, while *NbEIN2*-silenced plants showed reduced expression of *NbEAS* (Fig. 4B). Second, INF1-induced ethylene production was quantified in control, *NbEIN2*-, and *NbNup75*-silenced plants. At 10 h after treatment with INF1, treated leaf discs were sealed in gas chromatography (GC) vials for 3 h, and the produced ethylene was measured by GC. The amount of ethylene produced by INF1 treatment in *NbNup75*-silenced plants was reduced compared with the control and *NbEIN2*-silenced plants (Fig. 4C), suggesting that *NbNup75* is involved in ethylene production induced by INF1. INF1-induced expressions of most genes for enzymes

Fig. 3. Effects of gene silencing of *NbNup75*, *NbNup88*, or *NbSeh1* on INF1-induced defense responses. **A**, Effect of gene silencing of *NbNup75*, *NbNup88*, or *NbSeh1* on INF1-induced O_2^- production. Leaves of Tobacco rattle virus (TRV) infected *Nicotiana benthamiana* were treated with H_2O (left panel), or TRV-infected or gene-silenced *N. benthamiana* were treated with 150 nM INF1 (right panels). Production of O_2^- was detected as L-012 mediated chemiluminescence 12 h after the treatment. Circles indicate the area of treatment. Chemiluminescence images were obtained using a CCD camera. **B**, Intensities of chemiluminescence were quantified with photon image processor. Data are means $\pm$ standard error (SE) ($n = 15$). Results shown are representative of at least three separate experiments. **C**, *NbNup75* and *NbSeh1* are involved in the production of phytoalexin induced by INF1. Phytoalexin was extracted 24 h after 150 nM INF1 treatment and quantified by high-performance liquid chromatography. Data are means $\pm$ SE ($n = 3$). Results shown are representative of at least three separate experiments. **D**, Expression of *NbEAS* was repressed in *NbNup75*-silenced *N. benthamiana*. Total RNA was isolated from gene-silenced or control *N. benthamiana* 9 h after treatment with water (H_2O) or 150 nM INF1 (INF1). Quantitative reverse-transcriptase polymerase chain reaction was performed with *NbEAS* gene-specific primers. Expression levels of *NbEAS* were quantified relative to that of constitutively expressing *NbEF-1 α* . Data are means $\pm$ SE ($n = 3$). Results shown are representative of at least three separate experiments. Data marked with asterisks are significantly different from control (TRV) as assessed by the two-tailed Student's *t* test: * and ** indicate $P < 0.05$ and 0.01 , respectively.

in the mevalonate pathway were suppressed in *NbNup75*-silenced plants (Fig. 4D). Collectively, these results suggest that *NbNup75* is required for ethylene production but not for the response to ethylene during the induction of defense responses.

***NbNup75* is involved in INF1-induced expression of ACS genes.**

Activation of ACS is known as a rate-limiting step for the induced production of ethylene (Chae et al. 2003). Analysis of the *N. benthamiana* draft genome sequence identified 10

NbACS genes (Supplementary Fig. S7). In all, 7 of 10 *NbACS* genes have more than two highly homologous copies (data not shown), probably due to the allopolyploid nature of the *N. benthamiana* genome. Expression analysis of the identified 10 *NbACS* genes revealed that expression of *NbACS1*, *NbACS2*, and *NbACS4* was induced by INF1 treatment (data not shown). INF1-induced expression of *NbACS1*, *NbACS2*, and *NbACS4* was compared between control and *NbNup75*-silenced *N. benthamiana*. Expression of all tested *NbACS* genes was significantly reduced in *NbNup75*-silenced plants (Fig. 5).

NbNup75 is required for resistance reactions induced via the NbMEK2-NbSIPK/NbWIPK cascade.

Previously, it was reported that transient expression of NtMEK2^{DD}, a constitutive active form of MAPK kinase, induces ethylene production in *Arabidopsis* (Kim et al. 2003). To investigate the role of MAPK cascade in INF1-induced ethylene production, NbSIPK and NbWIPK, the downstream MAPK of NbMEK2, were employed. In *NbSIPK*- and *NbWIPK*-silenced plants, the production of ethylene induced by INF1 was approximately 30 to 50% compared with that of control plants, and there was no induction of ethylene production in *NbSIPK/NbWIPK*-silenced plants (Supplementary Fig. S8A). Similarly, *Agrobacterium*-mediated expression of NbMEK2^{DD} induced ethylene production in control *N. benthamiana*, and NbMEK2^{DD}-induced ethylene production was reduced by silencing of either *NbSIPK* or *NbWIPK* and almost abolished in *NbSIPK/NbWIPK*-silenced plants. In *NbNup75*-silenced *N. benthamiana*, ethylene production induced by expression of NbMEK2^{DD} was reduced (Fig. 6A). Moreover, NbMEK2^{DD}-induced as well as INF1-induced cell death was delayed in *NbNup75*-silenced *N. benthamiana* (Fig. 6B and C; Supplementary Fig. S9). These data indicated that NbNup75 is required for resistance reactions induced via the NbMEK2-NbSIPK/NbWIPK cascade.

Phosphorylation and nuclear localization of MAPK were not affected by the silencing of NbNup75.

The effect of *NbNup75*-silencing on phosphorylation of NbWIPK and NbSIPK was investigated using an antibody for phosphorylated MAPK (Katou et al. 2005). Within 60 min after the INF1 treatment, transient phosphorylation of NbSIPK and NbWIPK was detected. However, there was no obvious difference on phosphorylation of MAPK between control and *NbNup75*-silenced plants (Fig. 7A). Because previous reports indicated the increased nuclear localization of MAPK by the induction of disease resistance and ozone exposure (Ahlfors et al. 2004; Lee et al. 2004), we expected that nuclear-cytoplasmic transport of MAPK could be affected by the silencing of *NbNup75*. However, particle bombardment-based transient ex-

pression of green fluorescent protein (GFP)-labeled NbSIPK and NbWIPK revealed that there was no significant difference on cellular distribution of MAPK between control and *NbNup75*-silenced plants (Fig. 7B). Nuclear localization of GFP-labeled NbWRKY8 (*Arabidopsis* WRKY33 ortholog), a transcription factor phosphorylated by NbSIPK and NbWIPK (Ishihama et al. 2011), was also not affected by the silencing of *NbNup75*. Nuclear accumulation of MAPK after INF1 treatment was further assessed using MAPK antibodies. There is no significant induction of nuclear accumulation of MAPK by INF1 treatment, and the amount of MAPK in the nuclear fraction was not affected by the silencing of *NbNup75* (Fig. 7C).

Increased nuclear accumulation of polyA RNA in NbNup75-silenced plant.

A conserved function of Nup107-160 complex is the export of mRNA from nuclei (Dong et al. 2006; Doye et al. 1994; Parry et al. 2006; Vasu et al. 2001). Poly(A) RNA in situ hybridization

Fig. 5. INF1-induced expressions of *NbACS* genes were suppressed in *NbNup75*-silenced *Nicotiana benthamiana*. Total RNA was isolated from *NbNup75*-silenced or control *N. benthamiana* 12 h after treatment with water (H₂O) or 150 nM INF1. Quantitative reverse-transcriptase polymerase chain reaction was performed with gene-specific primers. Expression levels of genes were quantified relative to that of constitutively expressing *NbEF-1α*. Results shown are representative of at least three separate experiments. Data are means ± standard error (*n* = 4). Data marked with asterisks are significantly different from control (TRV) treated with INF1 as assessed by the two-tailed Student's *t* test: * and ** indicate *P* < 0.05 and 0.01, respectively.

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Fig. 4. NbNup75 is involved in expression of genes for phytoalexin production via ethylene (ET) biosynthesis. **A**, Biosynthetic pathway of sesquiterpenoid phytoalexins in *Nicotiana benthamiana*. Expression of some genes for phytoalexin biosynthesis was upregulated by ET treatment. Total RNA was isolated from *Tobacco rattle virus* (TRV)-infected *N. benthamiana* exposed to 100 ppm ET for 6 h. Numerical values shown in the pathway indicate relative gene expression in ET-treated leaves to that of mock-treated control. Constitutively expressing *NbEF-1α* was used as an internal standard. Data are means ± standard error (SE) (*n* = 3). Results shown are representative of at least three separate experiments. **B**, NbNup75 is not required for ET-induced expression of *NbEAS*. *NbNup75*-, *NbEIN2*-silenced, or control *N. benthamiana* exposed to 100 ppm ET for 6 h. Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with *NbEAS* gene-specific primers. Expression levels of *NbEAS* are quantified relative to that of constitutively expressing *NbEF-1α*. Data are means ± SE (*n* = 3). Data marked with asterisks are significantly different from control (TRV) treated with ET as assessed by the two-tailed Student's *t* test: * indicates *P* < 0.05. Results shown are representative of at least three separate experiments. **C**, ET production induced by INF1 was suppressed in *NbNup75*-silenced plant. Leaves of control or *NbNup75*- or *NbEIN2*-silenced *N. benthamiana* were treated with 150 nM INF1 and leaf disks were kept in a gas chromatography (GC) vial from 10 h after treatment. ET production in the following 3 h was quantified by GC. Data are means ± SE (*n* = 3). Data marked with asterisks are significantly different from control (TRV) treated with INF1 as assessed by the two-tailed Student's *t* test: * indicates *P* < 0.05. Results shown are representative of three separate experiments. **D**, INF1-induced expression of genes for phytoalexin biosynthesis was suppressed in *NbNup75*-silenced *N. benthamiana*. Total RNA was isolated from *NbNup75*-silenced or control *N. benthamiana* 9 h after treatment with water (H₂O) or 150 nM INF1 (INF1). RT-PCR was performed with gene-specific primers. Constitutively expressing *NbEF-1α* was used as an internal standard. Data are means ± SE (*n* = 3). Results shown are representative of at least three separate experiments. Data marked with asterisks are significantly different as assessed by the two-tailed Student's *t* test: * and ** indicate *P* < 0.05 and 0.01, respectively.

was performed to investigate the effect of *NbNup75* silencing on mRNA accumulation in nuclei using an Alexa488-labeled oligo(dT)45 probe. A stronger fluorescence signal inside nuclei was observed in some cells of *NbNup75*-silenced plants in contrast to relatively weak fluorescence in control plants, indicating the enhanced nuclear accumulation of mRNA in *NbNup75*-silenced plants (Fig. 8). Similar results were obtained for leaf tissues treated with either water or INF1 (data not shown).

Expression at later but not earlier time point of biphasic *NbWIPK* induction was affected by the silencing of *NbNup75*.

The expression profile of *NbWIPK* and *NbSIPK* in *N. benthamiana* treated with INF1 was investigated. Expression of *NbSIPK* was not induced by INF1 treatment, whereas that of *NbWIPK* was increased, with two peaks at 1 and 12 h after INF1 treatment (Supplementary Fig. S10). Increased expression of *NbWIPK* at 12 h was reduced in *Nup75*-silenced plant, whereas induction of *NbWIPK* at 1 h after the treatment was not affected by the silencing of *NbNup75* (Fig. 9). A similar biphasic expression pattern was observed for *NbWRKY8*. Increased expression of *NbWRKY8* and *NbACS4* at the later stage of defense induction was also reduced, as seen in the case of *NbWIPK* (Fig. 9).

DISCUSSION

Plant cells transcriptionally regulate a large number of genes to cope with diverse abiotic and biotic stresses. After the detection of an alert signal from the environment, one of the key steps in the control of gene expression is probably the transport of regulatory molecules between cytoplasm and nucleus. In eukaryotic cells, the nucleo-cytoplasmic exchange of proteins and RNAs is mediated through nuclear pores in the nuclear envelope. The NPC is composed of approximately 30 nucleoporins, with the basal structure of the NPC conserved among vertebrate, yeast, and plant (Neumann et al. 2010). Three main subcomplexes—Nup62 (central channel), Nup107-160 (outer rings), and Nup93 (inner rings)—are common in the architecture of eukaryote NPC, and the amino acid sequences of nucleoporins composing each subcomplex retains relatively high homology among different species (Tamura et al. 2010). Although the functional and structural analyses of nucleoporins have been carried out in vertebrate and yeast, little is known about the function of plant nucleoporins.

NbNup75 is not required for normal plant growth but is essential for the induction of disease resistance in *N. benthamiana*.

In this study, we identified *NbNup75* as an essential factor for the resistance of *N. benthamiana* against *P. infestans*. Vertebrate Nup75 and yeast Nup85p are components of the Nup107-160 nuclear pore subcomplex (yeast Nup84 complex), which is located on both the cytoplasmic and nuclear sides of the nuclear pore (Grossman et al. 2012). Based on the structural study, Nup75 is a component of Y-shaped heptameric complex, which assembled to form the outer ring of the NPC (Kampmann and Blobel 2009). Despite the fact that the Y-

Fig. 6. Silencing of *NbNup75* compromised NbMEK2-induced ethylene production and cell death. **A**, Silencing of *NbNup75* compromised NbMEK2-induced ethylene production. Leaves of control or *NbNup75*-silenced *Nicotiana benthamiana* were inoculated with *Agrobacterium tumefaciens* with pNPP40 or pNPP40-NbMEK2^{DD} and leaf disks were kept in a gas chromatography (GC) vial from 36 h after inoculation. Ethylene production in the following 3 h was quantified by GC. Data are means $\pm$ standard error (SE) ($n = 3$). Results shown are representative of three separate experiments. Data set marked with asterisk is significantly different as assessed by the two-tailed Student's *t* test: * indicates $P < 0.05$. **B**, NbMEK2-induced cell death was delayed in *NbNup75*-silenced plant. Leaves of control or *NbNup75*-silenced plants were stained with lactophenol trypan blue 2 days after agroinfiltration to visualize hypersensitive response-like cell death. Results shown are representative of three separate experiments. **C**, NbMEK2^{DD}-dependent cell death was quantified by ion leakage from eight leaf disks (1 cm in diameter) in 1 ml of water at 48 h after agroinfiltration. Data are means $\pm$ SE ($n = 3$). Results shown are representative of at least three separate experiments. Data set marked with asterisk is significantly different as assessed by the two-tailed Student's *t* test: * and ** indicate $P < 0.05$ and 0.01 , respectively.

shaped structure is expected to be disrupted by the loss of Nup75, *NbNup75*-silenced *N. benthamiana* and *Arabidopsis* Nup75 mutants can grow almost normally under laboratory conditions (Wiermer et al. 2012), indicating that the loss of Nup75 has a minor effect on the structure of the NPC and regular expression of genes for vegetative growth. In contrast, silencing of *NbNup88* caused obvious growth defects, which is consistent with that of the null mutation of *Arabidopsis* Nup88 being lethal (Cheng et al. 2009). Although Nup88 is predicted as a component of cytoplasmic rings and filaments which are not the central structural module for NPC (Raices and D'Angelo

2012), silencing of *NbNup88* caused severe defects on the formation of nuclei and the shrinkage of leaves, indicating that Nup88 is required for the formation and fundamental function of nuclei.

Seh1 and Nup75 are two closely related nucleoporins, which directly interact in the Nup107-160 subcomplex (Groth et al. 2010; Kampmann and Blobel 2009). Both NbNup75 and NbSeh1 are required for the production of ROS and phytoalexin in *N. benthamiana*, indicating that they would function together for the induction of disease resistance. In *L. japonicas*, Nup85 (Nup75) and Seh1 (NENA) were identified as

Fig. 7. A, INF1-induced phosphorylation of mitogen-activated protein kinases (MAPKs) was not affected by the gene silencing of *NbNup75*. Leaves of control or *NbNup75*-silenced *Nicotiana benthamiana* were treated with water (H₂O) or 150 nM INF1 (INF1) and harvested 0, 30, 60, and 540 min after treatment. Total proteins were prepared from harvested leaves and phosphorylation of NbSIPK and NbWIPK were detected using anti-phospho-p44/42 MAPK antibody (anti-P-MAPK). Protein loads were monitored by Coomassie Brilliant Blue (CBB) staining of bands corresponding to the ribulose-1,5-bisphosphate carboxylase large subunit (RBCL). **B**, Subcellular localization of green fluorescent protein (GFP)-tagged SIPK, WIPK, and WRKY8 in *Tobacco rattle virus* (TRV)-infected or *NbNup75*-silenced *N. benthamiana*. GFP-tagged proteins were transiently expressed by particle bombardment and expressed GFP, GFP-NbSIPK, NbWIPK-GFP, and NbWRKY8-GFP in epidermal cells were monitored by fluorescence confocal microscopy 18 h after bombardment. Bars = 30 μ m. **C**, Nuclear distribution of MAPKs was not affected by the gene silencing of *NbNup75*. Leaves of control or *NbNup75*-silenced *N. benthamiana* were treated with 150 nM INF1 and harvested 0, 1, 3, and 6 h. Total or nuclear protein were prepared from harvested leaves and NbSIPK and NbWIPK were detected using specific antibodies. Protein loads were monitored by CBB staining. Arrowhead indicates bands corresponding to the RBCL.

required factors for the establishment of symbiosis with both rhizobial bacteria and mycorrhizal fungi (Groth et al. 2010; Saito et al. 2007). In contrast, a recent study using a reverse genetics approach targeted to the Nup107-160 subcomplex revealed that *Arabidopsis* Seh1 but not Nup75 was required for

basal and R-protein-dependent defense against bacterial and oomycete pathogens (Wiermer et al. 2012). Such diverse requirement of Nup components in different plant-microbe interactions would indicate that each Nup in the Nup107-160 subcomplex forms not a simple structural module; instead, each

Fig. 8. Distribution of Poly(A) RNA in mesophyll cells of control and *NbNup75*-silenced *Nicotiana benthamiana*. Left: Leaves of control (*Tobacco rattle virus* [TRV]) and *NbNup75*-silenced *N. benthamiana* were fixed and probed with Alexa488-tagged oligo(dT)₄₅. Images shown were representative data from at least 20 samples from three separate experiments. Bars = 30 μ m. Right: Kymographs of Alexa488 fluorescence in mesophyll cells of control and *NbNup75*-silenced *N. benthamiana* created from images shown in left panels.

Fig. 9. Expression of *NbWIPK*, *NbWRKY8*, and *NbACS4* but not *NbSIPK* was repressed in *NbNup75*-silenced plant. Total RNA was isolated from *NbNup75*-silenced or *Tobacco rattle virus* (TRV)-infected *Nicotiana benthamiana* 0, 1, 3, 6, and 12 h after treatment with 150 nM INF1. Expression levels of genes were quantified relative to that of constitutively expressing *NbEF-1 α* . Data are means $\pm$ standard error ($n = 3$). Data marked with asterisks are significantly different from control (TRV) treated with INF1 as assessed by the two-tailed Student's *t* test: * and ** indicate $P < 0.05$ and 0.01, respectively. Results shown are representative of three separate experiments.

has a distinct role in the selective nucleocytoplasmic transport of molecules.

NbNup75 is not required for the activation of SIPK/WIPK and expression of defense genes at an early stage of defense induction.

Expression of *AtACS2* and *AtACS6*, stress-induced *ASC* genes of *Arabidopsis*, are regulated by the MKK4/MKK5-MPK3/MPK6 cascade (Han et al. 2010; Liu and Zhang 2004). A recent report indicated that *Arabidopsis* WRKY33, a substrate of MPK3/MPK6, directly binds to the W-boxes in the promoter regions of *AtACS2* and *AtACS6* to upregulate their transcription (Li et al. 2012). In this study, it was shown that the NbMEK2-NbSIPK/WIPK cascade, an orthologous MAPK cascade of *Arabidopsis* MKK4/MKK5-MPK3/MPK6, was an upstream regulator of ethylene production during disease resistance. *N. benthamiana* WRKY8, a homolog of *Arabidopsis* WRKY33, has been identified as a substrate of SIPK and WIPK (Ishihama et al. 2011). By analogy with *Arabidopsis* research, it was expected that WRKY8 transcription factors would be involved in the transcriptional upregulation of *NbACS* genes downstream of the NbMEK2-NbSIPK/WIPK cascade.

We initially predicted that NbNup75 regulates nucleocytoplasmic transport of downstream factors of NbMEK2, such as SIPK, WIPK, and WRKY8. There are several previous reports showing the translocation of plant MAPK from cytosol to nucleus during stress responses (Ahlfors et al. 2004; Lee et al. 2004). However, our testing for the distribution of SIPK and WIPK failed to prove the translocation of MAPK to the nucleus in *N. benthamiana* after INF1 treatment (Fig. 7C). Also, we did not detect any significant difference in cytoplasmic or nuclear distribution of SIPK, WIPK, and WRKY8 between control and *NbNup75*-silenced plants (Fig. 7B and C). Given that neither phosphorylation of NbSIPK/NbWIPK nor induction of defense genes within 1 h after INF1 treatment was affected by the silencing of *NbNup75*, Nup75 is not involved in the primary signal transduction from perception of signal to gene expression, which probably was mediated by preexisting signaling factors. NbNup75 was also not required for the ethylene-induced expression of *NbEAS* (Fig. 4B). These results indicate that NbNup75 is not required for i) the production of signaling molecules for the initial induction of defense responses and ii) the nuclear transport of signaling molecules for the induction of defense genes (Supplementary Fig. S11).

How could the defect in nuclear export of mRNA affect the induction of disease resistance?

Similar to the yeast *Nup85* mutant and *Arabidopsis* *Nup160* and *Seh1* mutants (Goldstein et al. 1996; Wiermer et al. 2012), enhanced nuclear accumulation of mRNA was observed in *NbNup75*-silenced *N. benthamiana* (Fig. 8). It is interesting that some but not all of the nuclei showed mRNA accumulation, as in the case of *Arabidopsis* *Nup160* and *Seh1* mutants (Wiermer et al. 2012), which might indicate the functional diversity of plant mesophyll cells during vegetative growth.

Why does the defect of mRNA export in *NbNup75*-silenced plant have no major effect on the growth of plant as well as production of signaling factors for initial defense induction? One possible explanation would be that the loss of Nup75 causes the delay of mRNA export from nuclei but it might not be a critical problem for the productivity of proteins, which are encoded by constitutively expressing genes. In contrast, timely production of factors for disease resistance should be essential for the successful defense against pathogens. In *NbNup75*-silenced plants, expression of defense genes in the first phase

of signal transduction was comparable with the nonsilenced plant but ineffective export of mRNA could disturb the induction of large number of defense-related genes in later stage of defense responses (i.e., genes for capsidiol production).

The role of NbNup75 might not be specific for disease resistance, because the expression level of *NbACS1* in water-treated leaves was also reduced in *NbNup75*-silenced plants (Fig. 5). Expression of *NbACS1* but not *NbASC2* and *NbASC4* was induced by water treatment as well as INF1 (data not shown); therefore, NbNup75 might be required for the expression of broad range of genes induced during stress responses.

Concluding remarks.

In this study, we identified NbNup75 as an essential component for full resistance of *N. benthamiana* to *P. infestans*. Considering that signaling events for the early stage of defense induction were not affected by the silencing of *NbNup75*, Nup75 appeared not to be involved in the initial signal transduction from pathogen recognition to the expression of defense genes. Effect of *NbNup75*-silencing on later stages of the resistance reactions, including phytoalexin production, induction of cell death, and expression of defense genes, would indicate that Nup75 is required for the amplification of the defense signal for effective defense against pathogens. Given that the pre-treatment of capsidiol can partially complement the resistance of *NbNup75*-silenced plants to *P. infestans*, a defect of capsidiol production would be a major reason for reduced resistance of *NbNup75*-silenced plants. However, overall weakened resistance reactions (including ROS production and cell death induction) would be related to the compromised resistance of *NbNup75*-silenced plants. Enhanced nuclear accumulation of mRNA in *NbNup75*-silenced plants might compromise the production of signaling factors for the amplification of defense induction. However, there is no direct evidence to prove that the disturbed export of mRNA is the cause of reduced resistance of *NbNup75*-silenced plants. *Arabidopsis* *seh1* and *nup160* mutants showed the defect on mRNA export from nuclei and reduced resistance against bacterial pathogens but the *nup75* mutant was as resistant as the wild type to bacterial pathogen *Pseudomonas syringae* (Wiermer et al. 2012). If ineffective export of mRNA in *seh1* and *nup160* mutants is the cause of reduced disease resistance of these mutants, it is a little contradictory that *Arabidopsis* *nup75* mutants showed full resistance to the pathogen in previous report.

In yeast cells, nucleoporins in the Nup84 complex are known to be involved in nuclear accumulation of the small GTPase Ran/Gsp1 (Gao et al. 2003). Prp20p, the nucleotide exchange factor for Ran, is mislocalized to the cytoplasm in yeast *nup85* mutant. Ran is involved in both import and export of proteins and mRNA across the nuclear envelope by interacting with karyopherins (importins and exportins) (Raices and D'Angelo 2012). Therefore, we can't exclude the possibility that nuclear-cytoplasmic transport of another factors besides mRNA is affected by the silencing of *NbNup75*. Moreover, attention needs to be paid to other possible contributions of nucleoporins in the regulation of gene expression, such as the regulation of genomic architecture and NPC-independent regulation of gene expression (Raices and D'Angelo 2012).

MATERIALS AND METHODS

Biological materials, growth conditions, and inoculation.

N. benthamiana (line SNPB-A5) was grown in an environmentally controlled growth room at 23°C with 16 h of light per day. *Phytophthora infestans* isolate 08YD1 (Shibata et al. 2011) was cultured on rye-media at 20°C. Inoculation of *N. benthamiana* leaves (approximately 45 days old) with zoospore sus-

pension of *P. infestans* was performed as previously described (Shibata et al. 2011).

Construction of vectors and VIGS.

Base vectors, primer sets, and template for the polymerase chain reaction (PCR) amplification of integrated DNA fragments and restriction sites used for the construction of all vectors used in this study are listed in Supplementary Table S2. Induction of VIGS was performed as previously reported (Ratcliff et al. 2001; Shibata et al. 2010).

Transient expression of GFP-labeled MAPK and WRKY8 by particle bombardment.

Particle bombardments were carried out using a Bio-Rad PDS-1000/He biolistic particle delivery system (Bio-Rad, U.S.A.), as previously described (Takemoto et al. 2012). Expression vectors (1 µg) were used to coat 1.25 µdeg of 1-µm diameter gold particles in a 25-µl suspension. Transformed epidermal cells of *N. benthamiana* were monitored by confocal laser-scanning microscopy 24 h after bombardments. Confocal fluorescence images were recorded on an FV1000-D microscope (Olympus, Japan). The laser was used as a 488-nm excitation source, and GFP fluorescence was recorded between 495 and 520 nm.

Treatment of INF1 elicitor and expression of NbMEK2^{DD} by agroinfiltration.

Preparation and treatment of INF1 elicitor was performed as previously described (Shibata et al. 2010). Transient expression of NbMEK2^{DD} was performed by the agroinfiltration of *A. tumefaciens* carrying pNPP40- NbMEK2^{DD} by the method previously reported (Shibata et al. 2010).

Quantitative reverse-transcriptase PCR and reverse-transcriptase PCR.

Total RNAs were isolated from *N. benthamiana* leaves using TRIzol Reagent (Life Technologies, U.S.A.) and cDNA synthesis was conducted using a commercial kit (ReverTra Ace-α; Toyobo, Japan). Quantitative reverse-transcriptase PCR (qRT-PCR) analysis was performed using a Thermal Cycler Dice Real Time System Single TP800 (Takara, Japan) with Thunderbird SYBR qPCR Mix (Toyobo). Biomass of *P. infestans* was determined by qRT-PCR with specific primers for the *PiActin* gene (PITG_15117) and expression of *N. benthamiana NbEF-1α* gene was used as internal control. Semiquantitative RT-PCR was performed with GoTaq Master Mix (Promega Corp., U.S.A.). Gene-specific primers used for expression analysis were listed in Supplementary Table S3.

Quantitative analysis of ROS production, capsidiol accumulation, ethylene production, and induction of cell death.

The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence, as reported previously (Uruma et al. 2009). Quantification of capsidiol produced in leaves of *N. benthamiana* was performed as previously described (Matsukawa et al. 2013). Quantitative analysis of ethylene production was performed using a gas chromatograph GC-353 equipped with a flame thermionic detector (GL Sciences, Japan) and CP-PoraBOND Q column (Varian Inc., The Netherlands), as described previously (Matsukawa et al. 2013). Induction of cell death was quantified by measuring the ion leakage from leaf tissue after elicitor treatment. Eight leaf disks of *N. benthamiana* were excised with cork borer (1 cm in diameter) and gently shaken (120 rpm) for 3 h in 1 ml of water, and ion conductivity of collected water was determined using pH/Ion meter F-54 (Horiba, Japan).

Lactophenol trypan blue staining.

To visualize hypersensitive response-like cell death, leaves of *N. benthamiana* were stained with lactophenol trypan blue as described by Takemoto and associates (2003), with a minor modification. Briefly, leaves of *N. benthamiana* were incubated for 80 min at 65°C in modified lactophenol trypan blue stain (5 ml of lactic acid, 5 ml of glycerol, 5 g of phenol, and 10 mg of trypan blue in 50% ethanol). Leaves were destained in chloral hydrate at 3 mg/ml for 2 days with gentle shaking.

Preparation of protein samples and western blotting.

Protein samples were prepared from 500 mg of ground *N. benthamiana* leaf tissues in liquid nitrogen with 500 µl of extraction buffer (50 mM morpholinepropanesulfonic acid [pH 7.6], 0.5 M sorbitol, 10 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 100 mM NaF, and 100 µM Na₃VO₄). Supernatant after centrifugation at 16,000 × g at 4°C for 10 min was used for protein samples. Nuclear protein fractions were prepared with a Plant Nuclei Isolation/Extraction Kit (Sigma). Western blot analysis was performed as reported previously (Shibata et al. 2010), with antibodies against WIPK (Seo et al. 1995), SIPK (Seo et al. 2000), histone H3 (clone number MABI0301, Wako, Japan), and phosphorylated MAPK (Phospho-p44/42 MAPK Erk 1/2 Thr202/Tyr204 XP Rabbit mAb number 4370; Cell Signaling Technology, U.S.A.).

Poly(A) RNA in situ hybridization.

Poly(A) RNA in situ hybridization was performed as described by Germain and associates (2010) using a Alexa488-labeled oligo(dT)45 (Operon Biotechnology, Tokyo). Confocal fluorescence images were recorded on an FV1000-D microscope (Olympus) with a 488-nm excitation source, and Alexa-488 fluorescence was recorded between 500 and 600 nm.

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LITERATURE CITED

- Ahlfors, R., Macioszek, V., Rudd, J., Brosché, M., Schlichting, R., Scheel, D., and Kangasjärvi, J. 2004. Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in *Arabidopsis thaliana* during ozone exposure. *Plant J.* 40:512-522.
- Bailey, J. A., Burden, R. S., and Vincent, G. G. 1975. Capsidiol: An antifungal compound produced in *Nicotiana tabacum* and *Nicotiana glauca* following infection with tobacco necrosis virus. *Phytochemistry* 14:597.
- Bombarely, A., Rosli, H. G., Vrebalov, J., Moffett, P., Mueller, L. A., and Martin, G. B. 2012. A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. *Mol. Plant-Microbe Interact.* 25:1523-1530.
- Broekaert, W. F., Delaure, S. L., De Bolle, M. F., and Cammue, B. P. 2006. The role of ethylene in host-pathogen interactions. *Annu. Rev. Phytopathol.* 44:393-416.

- Chae, H. S., Faure, F., and Kieber, J. J. 2003. The *eto1*, *eto2*, and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. *Plant Cell* 15:545-559.
- Cheng, Y. T., Germain, H., Wiermer, M., Bi, D., Xu, F., García, A. V., Wirthmueller, L., Després, C., Parker, J. E., Zhang, Y., and Li, X. 2009. Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in *Arabidopsis*. *Plant Cell* 21:2503-2516.
- Chung, I. M., Park, M. R., Rehman, S., and Yun, S. J. 2001. Tissue specific and inducible expression of resveratrol synthase gene in peanut plants. *Mol. Cells* 12:353-359.
- Doke, N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiol. Plant Pathol.* 23:345-357.
- Dong, C. H., Hu, X., Tang, W., Zheng, X., Kim, Y. S., Lee, B. H., and Zhu, J. K. 2006. A putative *Arabidopsis* nucleoporin, AtNUP160, is critical for RNA export and required for plant tolerance to cold stress. *Mol. Cell. Biol.* 26:9533-9543.
- Doye, V., Wepf, R., and Hurt, E. C. 1994. A novel nuclear pore protein Nup133p with distinct roles in poly(A)⁺ RNA transport and nuclear pore distribution. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:6062-6075.
- Facchini, P. J., and Chappell, J. 1992. Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 89:11088-11092.
- Fry, W. 2008. *Phytophthora infestans*: The plant (and *R* gene) destroyer. *Mol. Plant Pathol.* 9:385-402.
- Gao, H., Sumanaweera, N., Bailer, S. M., and Stochaj, U. 2003. Nuclear accumulation of the small GTPase Gsp1p depends on nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p, and the acetyl-CoA carboxylase Acc1p. *J. Biol. Chem.* 278:25331-25340.
- Germain, H., Qu, N., Cheng, Y. T., Lee, E., Huang, Y., Dong, O. X., Gannon, P., Huang, S., Ding, P., Li, Y., Sack, F., Zhang, Y., and Li, X. 2010. MOS11: A new component in the mRNA export pathway. *PLoS Genet.* 6:e1001250.
- Goldstein, A. L., Snay, C. A., Heath, C. V., and Cole, C. N. 1996. Pleiotropic nuclear defects associated with a conditional allele of the novel nucleoporin Rat9p/Nup85p. *Mol. Biol. Cell.* 7:917-934.
- Goodin, M. M., Zaitlin, D., Naidu, R. A., and Lommel, S. A. 2008. *Nicotiana benthamiana*: Its history and future as a model for plant-pathogen interactions. *Mol. Plant-Microbe Interact.* 21:1015-1026.
- Grossman, E., Medalia, O., and Zwerg, M. 2012. Functional architecture of the nuclear pore complex. *Annu. Rev. Biophys.* 41:557-584.
- Groth, M., Takeda, N., Perry, J., Uchida, H., Dräxl, S., Brachmann, A., Sato, S., Tabata, S., Kawaguchi, M., Wang, T. L., and Parniske, M. 2010. NENA, a *Lotus japonicus* homolog of *Sec13*, is required for rhizodermal infection by arbuscular mycorrhizal fungi and rhizobia but dispensable for cortical endosymbiotic development. *Plant Cell* 22:2509-2526.
- Han, L., Li, G. J., Yang, K. Y., Mao, G., Wang, R., Liu, Y., and Zhang, S. 2010. Mitogen-activated protein kinase 3 and 6 regulate *Botrytis cinerea*-induced ethylene production in *Arabidopsis*. *Plant J.* 64:114-127.
- Ishigaki, E., Asamizu, T., Arisawa, M., and Kurosaki, F. 2004. Cloning and expression of calmodulin genes regulating phytoalexin production in carrot cells. *Biol. Pharm. Bull.* 27:1308-1311.
- Ishihama, N., Yamada, R., Yoshioka, M., Katou, S., and Yoshioka, H. 2011. Phosphorylation of the *Nicotiana benthamiana* WRKY8 transcription factor by MAPK functions in the defense response. *Plant Cell* 23:1153-1170.
- Kamoun, S., van West, P., de Jong, A. J., de Groot, K. E., Vleeshouwers, V. G. A. A., and Govers, F. 1997. A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol. Plant-Microbe Interact.* 10:13-20.
- Kampmann, M., and Blobel, G. 2009. Three-dimensional structure and flexibility of a membrane-coating module of the nuclear pore complex. *Nat. Struct. Mol. Biol.* 16:782-788.
- Katou, S., Karita, E., Yamakawa, H., Seo, S., Mitsuhashi, I., Kuchitsu, K., and Ohashi, Y. 2005. Catalytic activation of the plant MAPK phosphatase NtMKP1 by its physiological substrate salicylic acid-induced protein kinase but not by calmodulins. *J. Biol. Chem.* 280:39569-39581.
- Kim, C., Liu, Y., Thorne, E., Yang, H., Fukushige, H., Gassmann, W., Hildebrand, D., Sharp, R., and Zhang, S. 2003. Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. *Plant Cell* 15:2707-2718.
- Lee, J., Rudd, J. J., Macioszek, V. K., and Scheel, D. 2004. Dynamic changes in the localization of MAPK cascade components controlling pathogenesis-related (*PR*) gene expression during innate immunity in parsley. *J. Biol. Chem.* 279:22440-22448.
- Li, G., Meng, X., Wang, R., Mao, G., Han, L., Liu, Y., and Zhang, S. 2012. Dual-level regulation of ACC synthase activity by MPK3/MPK6 cascade and its downstream WRKY transcription factor during ethylene induction in *Arabidopsis*. *PLoS Genet.* 8:e1002767.
- Liu, Y., and Zhang, S. 2004. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. *Plant Cell* 16:3386-3399.
- Matsukawa, M., Shibata, Y., Ohtsu, M., Mizutani, A., Mori, H., Wang, P., Ojika, M., Kawakita, K., and Takemoto, D. 2013. *Nicotiana benthamiana* Calreticulin 3a is required for the ethylene-mediated phytoalexins production and disease resistance against oomycete pathogen *Phytophthora infestans*. *Mol. Plant-Microbe Interact.* 26:880-892.
- Nakazato, Y., Tamogami, S., Kawai, H., Hasegawa, M., and Kodama, O. 2000. Methionine-induced phytoalexin production in rice leaves. *Biosci. Biotechnol. Biochem.* 64:577-583.
- Neumann, N., Lundin, D., and Poole, A. M. 2010. Comparative genomic evidence for a complete nuclear pore complex in the last eukaryotic common ancestor. *PLoS One* 5:e13241.
- Parry, G., Ward, S., Cernac, A., Dharmasiri, S., and Estelle, M. 2006. The *Arabidopsis* SUPPRESSOR OF AUXIN RESISTANCE proteins are nucleoporins with an important role in hormone signaling and development. *Plant Cell* 18:1590-1603.
- Raices, M., and D'Angelo, M. A. 2012. Nuclear pore complex composition: A new regulator of tissue-specific and developmental functions. *Nat. Rev. Mol. Cell Biol.* 13:687-699.
- Ralston, L., Kwon, S. T., Schoenbeck, M., Ralston, J., Schenk, D. J., Coates, R. M., and Chappell, J. 2001. Cloning, heterologous expression, and functional characterization of 5-*epi*-aristolochene-1,3-dihydroxylase from tobacco (*Nicotiana tabacum*). *Arch. Biochem. Biophys.* 393:222-235.
- Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. 2001. Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* 25:237-245.
- Ren, D., Liu, Y., Yang, K. Y., Han, L., Mao, G., Glazebrook, J., and Zhang, S. 2008. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 105:5638-5643.
- Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczygłowski, K., Downie, J. A., Parniske, M., Hayashi, M., and Kawaguchi, M. 2007. NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. *Plant Cell* 19:610-624.
- Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. 1995. Tobacco MAP kinase: A possible mediator in wound signal transduction pathways. *Science* 270:1988-1992.
- Seo, S., Okamoto, M., Seto, H., Iwai, T., Iwano, M., Fukui, K., Isogai, A., Nakajima, N., and Ohashi, Y. 2000. Reduced levels of chloroplast FtsH protein in *Tobacco mosaic virus*-infected tobacco leaves accelerate the hypersensitive reaction. *Plant Cell* 20:1988-1992.
- Shibata, Y., Kawakita, K., and Takemoto, D. 2010. Age-related resistance of *Nicotiana benthamiana* against hemibiotrophic pathogen *Phytophthora infestans* requires both ethylene- and salicylic acid-mediated signaling pathways. *Mol. Plant-Microbe Interact.* 23:1130-1142.
- Shibata, Y., Kawakita, K., and Takemoto, D. 2011. *SGT1* and *HSP90* are essential for age-related non-host resistance of *Nicotiana benthamiana* against the oomycete pathogen *Phytophthora infestans*. *Physiol. Mol. Plant Pathol.* 75:120-128.
- Takemoto, D., Jones, D. A., and Hardham, A. R. 2003. GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens. *Plant J.* 33:775-792.
- Takemoto, D., Rafiqi, M., Hurley, U., Lawrence, G. J., Bernoux, M., Hardham, A. R., Ellis, J. G., Dodds, P. N., and Jones, D. A. 2012. N-terminal motifs in some plant disease resistance proteins function in membrane attachment and contribute to disease resistance. *Mol. Plant-Microbe Interact.* 25:379-392.
- Tamura, K., Fukao, Y., Iwamoto, M., Haraguchi, T., and Hara-Nishimura, I. 2010. Identification and characterization of nuclear pore complex components in *Arabidopsis thaliana*. *Plant Cell* 22:4084-4097.
- Torres, M., and Dangl, J. L. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* 8:397-403.
- Tsuchisaka, A., and Theologis, A. 2004. Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. *Plant Physiol.* 136:2982-3000.
- Uruma, S., Shibata, Y., Takemoto, D., and Kawakita, K. 2009. *N,N*-dimethylsphingosine, an inhibitor of sphingosine kinase, induces phyto-

- alexin production and hypersensitive cell death of Solanaceae plants without generation of reactive oxygen species. *J. Gen. Plant Pathol.* 75:257-266.
- van Loon, L. C., Geraats, B. P., and Linthorst, H. J. 2006. Ethylene as a modulator of disease resistance in plants. *Trends Plant Sci.* 11:184-191.
- van Wees, S. C., Chang, H. S., Zhu, T., and Glazebrook, J. 2003. Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. *Plant Physiol.* 132:606-617.
- Vasu, S., Shah, S., Orjalo, A., Park, M., Fischer, W. H., and Forbes, D. J. 2001. Novel vertebrate nucleoporins Nup133 and Nup160 play a role in mRNA export. *J. Cell Biol.* 155:339-354.
- Wang, K. L., Li, H., and Ecker, J. R. 2002. Ethylene biosynthesis and signaling networks. *Plant Cell* 14 (Suppl.):S131-151.
- Wiermer, M., Cheng, Y. T., Imkampe, J., Li, M., Wang, D., Lipka, V., and Li, X. 2012. Putative members of the *Arabidopsis* Nup107-160 nuclear pore sub-complex contribute to pathogen defense. *Plant J.* 70:796-808.
- Yang, S. F., and Hoffman, N. E. 1984. Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* 35:155-189.
- Zarembinski, T. I., and Theologis, A. 1994. Ethylene biosynthesis and action: A case of conservation. *Plant Mol. Biol.* 26:1579-1597.