Age-Related Resistance of *Nicotiana benthamiana* Against Hemibiotrophic Pathogen *Phytophthora infestans* Requires Both Ethylene- and Salicylic Acid–Mediated Signaling Pathways

Yusuke Shibata, Kazuhito Kawakita, and Daigo Takemoto

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

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Phytophthora infestans, the agent of late blight disease of potato, is a hemibiotrophic pathogen with biotrophic action during early infection and necrotrophic in the later stage of colonization. Mature *Nicotiana benthamiana* was resistant to *P. infestans*, whereas relatively young plants were susceptible to this pathogen. Young plants became resistant following a pretreatment with acibenzolar-*S*-methyl, a functional analog of salicylic acid (SA), indicating that susceptibility of young plants is due to a lack of induction of SA signaling. Further analysis with virus-induced gene silencing indicated that *NbICS1* and *NbEIN2*, the genes for SA biosynthesis and ethylene (ET) signaling, respectively, are required for the resistance of mature *N. benthamiana* against *P. infestans*. Furthermore, these genes are required for the production of reactive oxygen species (ROS) induced by treatment of the INF1 elicitor. In *NbICS1*-silenced plants, cell death induced by either INF1 or necrosis-inducing protein NPP1.1 was significantly accelerated. Expression of genes for phytoalexin (capsidiol) biosynthesis, *NbEAS* and *NbEAH*, were regulated by ET, and gene silencing of either of them compromised resistance of *N. benthamiana* to *P. infestans*. Together, these results suggest that resistance of *N. benthamiana* against hemibiotrophic *P. infestans* requires both SA-regulated appropriate induction of cell death and ET-induced production of phytoalexin.

The oomycete pathogen, *Phytophthora infestans*, is the causal agent of potato late blight, which is one of the most destructive and economically important plant diseases worldwide. *P. infestans* is historically significant as a cause of the Irish potato famine in the 1840s, and this pathogen still causes a 10 to 15% reduction in the annual global potato production (CIP 1996). More than 10 dominant race-specific resistance (*R*) genes have been discovered in the wild species *Solanum demissum*, and some of these were introgressed into potato cultivars. However, *R*-gene-dependent resistances were quickly overcome by emerging new isolates of the pathogen (Umaerus and Umaerus 1994). Relatively recently, *R* genes conferring

broad-spectrum resistance to *P. infestans* have been identified from several wild *Solanum* spp., such as *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2005) and *Rpi-pnt1* from *S. pinnatisectum* (Kuhl et al. 2001). Introduction of these resistance to *P. infestans* (*Rpi*) genes into cultivated potato lines is considered the most practical approach to achieving durable resistance of potato plants against *P. infestans*.

Race-specific and broad-spectrum *R* genes generally induce a similar set of resistance reactions in response to a pathogen attack involving corresponding avirulence factors. These responses include production of physical and chemical barriers at the site of pathogen attack, accumulation of antimicrobial proteins and secondary metabolites, and the induction of hypersensitive cell death, a form of plant-programmed cell death that confines the attacking pathogen to the site of infection (Hahlbrock et al. 2003; Jones and Takemoto 2004). Production of reactive oxygen species (ROS) is characteristic of plant resistance reactions, and they are thought to act as second messengers for the induction of resistance reactions, such as the expression of defense genes and induction of hypersensitive cell death (or hypersensitive reactions [HR]) (Doke et al. 1996; Yoshioka et al. 2003; Yoshie et al. 2005). Induction of disease resistance is generally accompanied by activation of several signal transduction pathways mediated by “phytohormones” such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Glazebrook 2005). Extensive research using the Brassicaceous model plant *Arabidopsis thaliana* with various pathogens revealed that resistances to biotrophic and necrotrophic pathogens require different sets of defense reactions to effectively prevent their infection and colonization. *Arabidopsis* mutants with impaired SA signaling (i.e., *sid2* and *eds5*) failed to induce effective resistance against the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis*, whereas these mutants were totally resistant to necrotrophic *Alternaria brassicicola* and *Botrytis cinerea* (Nawrath and Métraux 1999; Ferrari et al. 2003; van Wees et al. 2003). In contrast, mutation of JA signaling (i.e., *coi1*) or phytoalexin (camalexin) production (i.e., *pad3*) compromised resistance to necrotrophic pathogens, whereas these mutants retained resistance to biotrophic *H. arabidopsidis* infection (Glazebrook et al. 1997; Thomma et al. 1999; Ferrari et al. 2003; van Wees et al. 2003). Based on these reports, it is generally considered that SA signaling is involved in resistance to biotrophic pathogens, whereas JA signaling is essential to stop infection by necrotrophic pathogens (McDowell and Dangl 2000; Glazebrook 2005).

Corresponding author: Daigo Takemoto; Telephone: +81 52 789 4029; Fax: +81 52 789 5525; E-mail: dtakemo@agr.nagoya-u.ac.jp

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P. infestans is a hemibiotrophic plant pathogen; it behaves as a biotrophic pathogen in the early stage of infection but acts as a necrotrophic pathogen in the late stage of colonization. Despite the practical importance of this pathogen, signaling pathways required for plant resistance against *P. infestans* are largely unknown, because of the lack of an appropriate plant model to investigate host–pathogen interactions. In artificial interactions between *Arabidopsis* and *P. infestans*, most of the penetration attempts by *P. infestans* ceased at the surface of the plant cell. *Arabidopsis pen2* and *pen3* mutants inoculated with *P. infestans* showed reduced penetration resistance accompanied with hypersensitive cell death at the site of infection. This indicated that penetration resistance is a major factor for the resistance of *Arabidopsis* against inappropriate pathogen *P. infestans*, which is supported by a post-invasion defense mechanism (Lipka et al. 2005; Kobae et al. 2006; Stein et al. 2006).

P. infestans is a pathogen for *Solanaceous* potato and tomato but most of *Solanaceous* spp., except potato and tomato, are generally resistant to *P. infestans* infection. The wild *Nicotiana* sp., *Nicotiana benthamiana*, is a *Solanaceous* model plant which can be applied for functional analysis of genes by manipulating expression level of genes of interest using *Agrobacterium* sp.-mediated transient gene expression or virus-induced gene silencing (VIGS) (Ratcliff et al. 2001). *N. benthamiana* is a good candidate as a model plant to analyze resistance reactions of *Solanaceous* plant against *P. infestans*. However, there are contradictory reports about interaction between *P. infestans* and *N. benthamiana*. Some reports indicated that *N. benthamiana* is resistant to *P. infestans* (Kamoun et al. 1998; Yoshioka et al. 2003), whereas others reported that *N. benthamiana* is susceptible to this pathogen (Becktell et al. 2006; Asai et al. 2008). These conflicting observations could be a result of conditional differences in *P. infestans* inoculation or differences in age of *N. benthamiana* or *P. infestans* isolates used in these studies.

The objectives of this research were to i) clarify the nature of the interaction between *P. infestans* and *N. benthamiana*, ii) identify the essential signal transduction pathways for *N. benthamiana* resistance to *P. infestans* infection, and iii) identify the genes or phenomena regulated by signal transduction pathways required for resistance to *P. infestans*. This allows an overview of the basis of *N. benthamiana* resistance to infection by hemibiotrophic pathogen *P. infestans*.

RESULTS

Age-related resistance of *N. benthamiana* against *P. infestans*.

To investigate the nature of the interaction between *N. benthamiana* and *P. infestans*, *N. benthamiana* plants at different stages of growth were inoculated with *P. infestans* isolate PI0-1 (race 0). Within 5 days after the inoculation of *P. infestans*, relatively young plants (20 to 25 days from the date of sowing) showed water-soaked disease symptoms on inoculated leaves (Fig. 1A), and the disease lesions almost extended over entire leaves within 14 days. In contrast, mature *N. benthamiana* plants (35 to 40 days old) were completely resistant to *P. infestans* (Fig. 1A and 1C). Most of the 30-day-old plants were resistant and only occasionally showed disease symptoms (Fig. 1C). These results indicated that resistance of *N. benthamiana* plants against *P. infestans* is dependent on their age. The interaction between *P. infestans* and young or mature *N. benthamiana* was examined using light microscopy, after staining with lactophenol trypan blue of inoculated leaves (staining of plant cells with trypan blue is indicative of cell death). In the interaction between *P. infestans* and mature *N. benthamiana*, a few densely stained epidermal and mesophyll cells under the pene-

tration site of *P. infestans* were observed from 12 h after inoculation. By 48 h after inoculation, there was no detectable mycelium growth of *P. infestans* within and around clusters of approximately five dead mesophyll cells (Fig. 1D). On young leaves inoculated with *P. infestans*, most of the encysted zoospores and zoosporangia had germinated and developed appressorium-like swellings that formed preferentially over anticlinal walls between epidermal cells at 6 h after inoculation (Fig. 1E). By 24 h after inoculation, extensive growth of *P. infestans* hyphae in intercellular spaces of mesophyll cells, with formation of finger-like haustoria, was observed. Corruption of a large number of mesophyll cells was observed where there was massive infection at 36 and 48 h after inoculation (Fig. 1E), indicating that transition from the biotrophic to necrotrophic phase of *P. infestans* infection occurred between 24 and 36 h after inoculation in interaction with young *N. benthamiana*. We also applied six additional isolates of *P. infestans* to test the resistance of *N. benthamiana*. All tested *P. infestans* isolates produced disease symptoms on 20- to 25-day-old *N. benthamiana* plants but mature plants were resistant to all isolates (Y. Shibata and D. Takemoto, unpublished data). These results confirmed that *N. benthamiana* has age-related resistance against *P. infestans*, and there is probably no fundamental difference in pathogenicity between isolates of *P. infestans*. Consequently, for subsequent analyses, we studied the interaction of 35-day-old *N. benthamiana* plants and *P. infestans* isolate PI0-1 unless otherwise noted.

Both SA- and ET-mediated signaling are essential for resistance of *N. benthamiana* to *P. infestans*.

To investigate the role of signaling pathways on resistance of *N. benthamiana* to *P. infestans*, VIGS was applied to knock-down genes involved in SA-, ET-, or JA-mediated signaling pathways. Gene fragments of *N. benthamiana* homologues of *A. thaliana* SA induction-deficient 2/isochorismate synthase 1 (*SID2/ICS1*) (Wildermuth et al. 2001), nonexpresser of pathogenesis-related (*PR*) genes (*NPRI*) (Cao et al. 1997), colonatin insensitive 1 (*COI1*) (Xie et al. 1998), and ET-insensitive 2 (*EIN2*) (Alonso et al. 1999) were isolated by reverse-transcription polymerase chain reaction (RT-PCR) and cloned into the vector for *Tobacco rattle virus* (TRV)-mediated VIGS, pTV00 (Ratcliff et al. 2001). In leaves of *N. benthamiana*, expression of *NbICS1* and *NbNPRI* was induced by treatment with INF1, a secretory elicitor protein derived from *P. infestans*, while *NbCOI1* and *NbEIN2* showed constitutive expression (Supplementary Fig. 1) (Kamoun et al. 1997). Expression of these genes was silenced by VIGS, and gene-silenced *N. benthamiana* were inoculated with *P. infestans* isolate PI0-1. It was confirmed that expression of target genes was suppressed in VIGS-induced plants treated with INF1 (Fig. 2A). All gene-silenced plants showed normal growth compared with the control TRV-infected plant, although the *NbICS1*-silenced plant showed weak chlorosis. This was consistent with previous reports, because isochorismate is the precursor for the biosynthesis of phyloquinone as well as SA and, consequently, mutation of the *ICS1* gene causes a reduction in photosystem I (PSI) activity and chlorophyll accumulation (Gross et al. 2006, Catnot et al. 2008). Within 2 days after the inoculation, *NbICS1*- and *NbEIN2*-silenced plant started to show the development of visible disease symptoms on the inoculated area, and the expansion of lesions beyond the inoculated area occurred over the period of observation (Fig. 2B). In contrast, *NbNPRI*- and *NbCOI1*-silenced *N. benthamiana* showed complete resistance against *P. infestans*, comparable with the control TRV-infected plant. Extension of *P. infestans* hyphae in planta and dead plant cells around infection sites were visualized by staining with lacto-

phenol-trypan blue. At 48 h after inoculation, HR-like local death of plant epidermal and mesophyll cells was occasionally observed at infection sites of the control, *NbNPR1*-, and *NbCOII*-silenced *N. benthamiana* plants (Fig. 2C). In *NbICS1*- or *NbEIN2*-silenced *N. benthamiana* plants, the bulk of the dead mesophyll cells were obvious around the original site of infection; however, *P. infestans* hyphae near the edge of lesions were generally not enclosed by the dead plant cells (Fig. 2C). Reduced resistance of *NbICS1*- or *NbEIN2*-silenced plants was scored by visible development of disease symptoms (Fig. 2D). Both *NbICS1*- and *NbEIN2*-silenced plants showed a significant reduction in resistance against *P. infestans*, although we consistently observed that *NbEIN2*-silenced plants showed faster development of disease symptoms compared with *NbICS1*-silenced plants (Fig. 2D). These results indicated that both SA- and ET-signaling pathways are essential for *N. benthamiana* to prevent infection and colonization by *P. in-*

festans. We also produced a VIGS construct for phenylalanine ammonia lyase (*NbPAL*) because PAL is known to be involved in SA production by an alternative pathway, in which phenylalanine is converted to benzoic acid (BA) and, subsequently, BA is converted to SA (Mauch-Mani and Slusarenko 1996; Shah 2003). However, *NbPAL*-silenced plants showed resistance comparable with control plants, indicating that *NbPAL* is dispensable for the age-related resistance of *N. benthamiana* (Supplementary Fig. 2).

To examine the effect of SA on susceptibility of young *N. benthamiana*, 25-day-old plants pretreated with acibenzolar-*S*-methyl (ASM), a functional analog of SA, at 100 ppm were inoculated with *P. infestans*. Young plants pretreated with ASM showed complete resistance against *P. infestans* (Supplementary Fig. 3) (Vallad and Goodman 2004), suggesting that the lack of resistance in young *N. benthamiana* is a result of deficient induction of a SA-mediated signaling pathway.

Fig. 1. Age-related resistance of *Nicotiana benthamiana* against *Phytophthora infestans*. **A**, *N. benthamiana* of different ages were tested for resistance to *P. infestans* isolate PI0-1. The numbers in each panel indicate the age of the plant in days from the date of sowing. Leaves of *N. benthamiana* were inoculated with a suspension of *P. infestans* zoospores at a concentration of 2×10^5 zoospore/ml and development of disease symptoms was evaluated. Results shown are representative of three separate experiments. Photographs of whole plants (top panels) and inoculated leaves (bottom panels) were taken 9 days after the inoculation. Arrowheads indicate the point of inoculation. **B**, Appearance of disease symptom showing differences in severity representative of the five classifications used in C. **C**, Plots showing percentages of *N. benthamiana* leaves with disease symptom severities in each of the five classes represented in B. Leaves of *N. benthamiana* of different ages were inoculated with *P. infestans* and severities of disease symptom were evaluated from 1 to 5 days after inoculation. At least 15 inoculated leaves from each plant of different ages were counted. **D** and **E**, Microscopic observation of mature (**D**) and young (**E**) *N. benthamiana* leaves after inoculation with *P. infestans*. Inoculated leaves were stained with lactophenol trypan blue to visualize dead plant cells and the hyphae of *P. infestans*. Black arrowheads and red arrows indicate the hyphae and haustoria of *P. infestans*, respectively; hpi, hours post inoculation; cy, cyst; as, appressorium-like swelling. Bars = 50 μ m.

***NbICS1*-silenced *N. benthamiana* showed enhanced cell death in response to the treatment of INF1 and NPP1.1.**

P. infestans produces a large number of secretory proteins during the process of infection, including intracellular effectors (e.g., RXLR effectors) (Oh et al. 2009) and apoplastic elicitors (e.g., INF1 and NPP1.1) (Kamoun et al. 1997, Fellbrich et al. 2002). INF1, a major secretory protein of *P. infestans*, is thought to be a sterol carrier, which enables the pathogen to uptake sterol from the host plant, and it is also considered to act as a semi-PAMP in oomycete pathogens recognized by a limited number of dicot plants, including *Nicotiana* spp.

(Kamoun et al. 1993; Blein et al. 2002; Takemoto et al. 2005). A previous report suggested that INF1 is a virulence factor in interaction between *P. infestans* and *N. benthamiana* (Kamoun et al. 1998). NPP1.1 is a member of the Nep1-like proteins (NLP), which induce nonspecific cell death in dicot plants, although the function of NPP1.1 is unknown (Kanneganti et al. 2006). Biological function of NPP1.1 is unknown. It was reported that INF1 and NPP1.1 induce the death of plant cells through distinct mechanisms (Kanneganti et al. 2006). We employed INF1 and NPP1.1 to examine the effect of gene silencing on the induction of plant cell death. We constructed chi-

Fig. 2. Both *NbICS1* and *NbEIN2* are essential for age-related resistance of *Nicotiana benthamiana* to *Phytophthora infestans*. **A**, *N. benthamiana* was inoculated with *Tobacco rattle virus* (TRV), TRV:ICS1, TRV:NPR1, TRV:COI1, or TRV:EIN2, and total RNA was isolated from *N. benthamiana* leaves 12 h after the treatment with 150 nM INF1. Silencing of target genes was confirmed by reverse-transcription polymerase chain reaction with gene-specific primers at least five separate leaves. **B**, Leaves of *NbICS1*-, *NbNPR1*-, *NbCOI1*-, or *NbEIN2*-silenced *N. benthamiana* were inoculated with *P. infestans* P10-1. Arrowheads indicate the point of inoculation. Photographs were taken 9 days after inoculation. **C**, Microscopic observation of *N. benthamiana* leaves 48 h after inoculation with *P. infestans*. Inoculated leaves were stained with lactophenol trypan blue to visualize dead plant cells and the hyphae of *P. infestans*. Arrowheads indicate the hyphae of *P. infestans*. Bars = 50 μ m. Similar results were obtained from at least 12 inoculated leaves. **D**, Plots showing percentages of *N. benthamiana* leaves with disease symptom severities in each of five classes. Leaves of *NbICS1*-, *NbNPR1*-, *NbCOI1*-, or *NbEIN2*-silenced plants were inoculated with *P. infestans* and severities of disease symptom were evaluated from 1 to 5 days after inoculation. At least 16 inoculated leaves from each silenced plant were counted.

meric transgenes for mature INF1 or NPP1.1 with the signal peptide (sp) of tobacco PR1a (Cornelissen et al. 1987) fused to the N terminus and with hemagglutinin (HA)/StrpII tags at the C terminus. This allowed for both the expression of mature elicitor proteins in the intercellular spaces of transformed plant tissue and the detection of expressed proteins. The INF1 or NPP1.1 constructs were expressed transiently under the con-

Fig. 3. *NbICS1*-silenced *Nicotiana benthamiana* showed enhanced induction of cell death after the expression of either INF1 or NPP1.1. **A**, Appearance of cell death showing differences in severity representative of the four classifications used in **B**. **B**, INF1:HA (hemagglutinin) (top) or NPP1:HA (bottom) were expressed by agroinfiltration in *NbICS1*-, *NbNPR1*-, *NbCOI1*-, or *NbEIN2*-silenced or control *N. benthamiana*. Plots show the severity of cell death among the *N. benthamiana* leaves and the percentages in each of the four classes represented in **A**. Leaves of *NbICS1*-, *NbNPR1*-, *NbCOI1*-, or *NbEIN2*-silenced or control plants were inoculated with *Agrobacterium tumefaciens* with INF1:HA (top) or NPP1:HA (bottom) and severities of cell death were evaluated from 1 to 4 days after inoculation. At least 30 inoculated leaves from each silenced plants were counted. **C**, Protein samples were extracted from leaves of *NbICS1*-, *NbNPR1*-, *NbCOI1*-, or *NbEIN2*-silenced plants and control plants 48 h after inoculation with *A. tumefaciens* with INF1:HA (top) or NPP1:HA (bottom). Expressed HA-tagged proteins were detected by immunoblot analysis with anti-HA antibody. Results shown are representative of at least three separate experiments.

trol of the *Cauliflower mosaic virus* (CaMV) 35S promoter by agroinfiltration into leaves of *N. benthamiana*. Cells of *N. benthamiana* leaves showed visible HR-like cell death induced by the expression of INF1, whereas there was no visible cell death after agroinfiltration with the vector alone (data not shown). Expression of NPP1.1 in the apoplastic space of *N. benthamiana* leaves also induced cell death, although cell death induced by NPP1.1 is considerably weaker compared with INF1-induced cell death (Fig. 3B). INF1 was expressed in leaves of *NbICS1*-, *NbNPR1*-, *NbCOI1*-, or *NbEIN2*-silenced plants and induction of cell death was scored until 5 days after the inoculation with *Agrobacterium tumefaciens* carrying the INF1 vector. *NbNPR1*, *NbCOI1*, and *NbEIN2* plants showed no significant difference in the induction of cell death compared with the TRV-infected control plant, whereas *NbICS1*-silenced plants showed accelerated induction of cell death (Fig. 3B). Similarly, expression of NPP1.1 in *ICS1*-silenced plants induced significantly more severe cell death (Fig. 3B). Immunoblot analysis was performed using anti-HA antibody to exclude the possibility that enhanced cell death in *NbICS1*-silenced plants was the result of increased accumulation of INF1 and NPP1.1 proteins. There were similar levels of the INF1 and NPP1.1 proteins in all gene-silenced and control plants (Fig. 3C); thus, it was confirmed that *NbICS1*-silenced plant showed enhanced induction of cell death.

Silencing of either *NbICS1* or *NbEIN2* compromised INF1-induced O_2^- production in *N. benthamiana*.

Production of ROS is a hallmark of plant defense responses and it is thought to control the degree of HR during the induction of disease resistance (Doke 1983; Torres and Dangl 2005).

Fig. 4. Effects of silencing of *NbICS1*, *NbNPR1*, *NbCOI1*, or *NbEIN2* genes on the O_2^- production induced by treatment with INF1. **A**, Leaves of *Tobacco rattle virus* (TRV)-infected control *Nicotiana benthamiana* was treated with water or 150 nM INF1, and production of O_2^- was detected as L-012 mediated chemiluminescence 12 h after treatment. Chemiluminescence images shown were obtained using a CCD camera. Circles indicate the area of water or INF1 treatment. **B**, Leaves of gene-silenced *N. benthamiana* were treated with 150 nM INF1 and production of O_2^- was detected as L-012 mediated chemiluminescence 12 h after treatment. **C**, Chemiluminescence intensities were quantified with a photon image processor. Data are means $\pm$ standard error from four separate experiments.

It has been reported that *N. benthamiana* produced ROS within 12 h after treatment with INF1 (Uruma et al. 2009). To investigate the effect of gene silencing on ROS production induced by INF1, *NbICS1*-, *NbNPR1*-, *NbCOI1*-, and *NbEIN2*-silenced *N. benthamiana* leaves were infiltrated with INF1, and production of ROS (O_2^-) was measured as the chemiluminescence using the luminol derivative L-012 (Nishinaka et al. 1993; Asai et al. 2008). At 12 h after treatment with INF1, *NbNPR1*- and *NbCOI1*-silenced *N. benthamiana* showed a level of ROS production similar to the control plant, whereas *NbICS1*- and *NbEIN2*-silenced *N. benthamiana* showed significant reduction of ROS production (Fig. 4A and B). Given that ROS production was reported to positively regulate the HR in disease resistance (Torres et al. 2002; Yoshioka et al. 2003; Pogány et al. 2009), *NbICS1*-silenced plants, which showed enhanced induction of cell death (Fig. 3), were expected to produce ROS rapidly in response to elicitor treatment. However, substantial production of ROS was not detected at 1, 3, or 6 h after INF1 treatment (data not shown); therefore, it is concluded that elicitor-induced ROS production was compromised in *NbICS1*-silenced plants. Together, these results indicated that both SA- and ET-mediated signaling pathways are necessary for the induction of INF1-induced ROS production in *N. benthamiana*.

Expression of defense-related genes in *ICS1*- and *EIN2*-silenced plants.

The effect of gene silencing of *NbICS1* or *NbEIN2* on the expression of various defense-related genes was investigated. Expression of *PR-1a*, a marker gene of disease resistance, is suppressed in *NbICS1*-silenced plant (Fig. 5), indicating that *NbPR-1a* is SA regulated, consistent with previous reports (Vlot et al. 2009). Vacuolar processing enzymes (VPE) were originally identified as enzymes involved in maturation of seed storage proteins (Hara-Nishimura et al. 1991). Recent reports also indicated that these enzymes have caspase activity required for the induction of the HR during the interaction between *Tobacco mosaic virus* (TMV) and tobacco plants with the corresponding resistance gene *N* (Hatsugai et al. 2004). Expression of *NbVPE* was significantly reduced in *NbICS1*-silenced plants (Fig. 5). 5-*Epi*-aristolochene synthase (EAS) is an enzyme for the biosynthesis of capsidiol, a major phytoalexin of *Nicotiana* and *Capsicum* spp. (Bailey et al. 1975). INF1-induced expression of *NbEAS* was repressed by gene silencing of *NbEIN2* (Fig. 5). Silencing of either *NbICS1* or *NbEIN2* had no effect on expression of marker gene *hsr203J*, which is specifically expressed in HR-induced cells (Pontier et al. 1998). Expression of *NbRbohB*, a gene for the respiratory-burst oxidase homolog (*rboh*) involved in ROS production during disease resistance (Asai et al. 2008; Yoshioka et al. 2003), was induced by INF treatment but not affected by silencing of either *NbICS1* or *NbEIN2*, although ROS production by these silenced plants was considerably reduced (Fig. 4). Therefore, it is suggested that regulation of ROS production by SA and ET signaling is post transcriptional.

Neither SA-regulated *NbPR-1a* nor *NbVPE* genes are involved in *N. benthamiana* resistance against *P. infestans*.

To investigate the role of SA-regulated genes in *N. benthamiana* resistance against *P. infestans*, *NbVPE*- and *NbPR-1a*-silenced *N. benthamiana* were inoculated with *P. infestans* (Supplementary Fig. 4). Over 10 days of observation after the inoculation, *NbVPE*- and *NbPR-1a*-silenced plants did not show any development of disease symptoms. Although the construct for silencing of *NbVPE* is designed to suppress the expression of two VPE genes, *NbVPE-1a* and *-1b*, which are reported to be involved in the *N*-gene-dependent cell death in-

duced by TMV (Hatsugai et al. 2004), *NbVPE*-silenced plants showed comparable induction of cell death after the treatment of INF1 to control plant (data not shown). Our data indicated that neither *NbPR-1a* nor the *NbVPE* genes are essential for *N. benthamiana* resistance against *P. infestans*.

Expression of genes for capsidiol biosynthesis, *NbEAS*, and *NbEAH*, are regulated by ET and essential for the resistance to *P. infestans*.

Nicotiana and *Capsicum* spp. produce a sesquiterpenoid phytoalexin, capsidiol, as a pathogen- and elicitor-inducible defense metabolite (Bailey et al. 1975). Formation of capsidiol involves the cyclization of farnesyl pyrophosphate (FPP) to the sesquiterpenoid hydrocarbon 5-*epi*-aristolochene catalyzed by EAS (Facchini and Chappell 1992) and two subsequent hydroxylation steps by 5-*epi*-aristolochene dihydroxylase (EAH) (Ralston et al. 2001). Given that INF1-induced expression of *NbEAS* was suppressed in *EIN2*-silenced *N. benthamiana*, it is expected that the induction of *NbEAS* expression is regulated by ET. We examined expression of *NbEAS* in *N. benthamiana* exposed to exogenous ET. At 6 h after the treatment, expression of *NbEAS* was significantly induced by treatment of ET at 100 ppm, consistent with suppressed expression of *NbEAS* in *EIN2*-silenced plants (Fig. 6A). Similarly, expression of *NbEAH* was also found to be induced by ET treatment (Fig. 6A). *NbEAS*- and *NbEAH*-silenced plants were produced to examine the role of capsidiol in age-related resistance of *N. benthamiana* to *P. infestans*. Within 5 days after the inoculation of *P. infestans*, *NbEAS*- and *NbEAH*-silenced plants showed severe disease symptoms, which were comparable with those of *NbEIN2*-silenced plants (Fig. 6C and E). Microscopic observation with lacto phenol trypan blue staining revealed extensive colonization by *P. infestans* (Fig. 6D). Remarkably, plant cells surrounding the extending hyphae of *P. infestans* were often well stained by trypan blue or collapsed, indicating that cells of *NbEAS*- or *NbEAH*-silenced plants were capable

Fig. 5. Expression of defense-related genes in *NbICS1*- or *NbEIN2*-silenced *Nicotiana benthamiana*. Total RNA was isolated from *NbICS1*- or *NbEIN2*-silenced or control *N. benthamiana* 12 h after treatment with water (H_2O) or 150 nM INF1 (INF1). Reverse-transcription polymerase chain reaction was performed with primers specific for *NbPR-1a*, *NbVPE*, *NbEAS*, *NbrbohB*, and *Nbhsr203J*. Constitutively expressing *NbEF-1α* was used as an internal standard. Results shown are representative of at least five separate experiments.

of inducing HR-like cell death in response to the contact with *P. infestans* hyphae (Fig. 6D). Consistently, induction of cell death and production of ROS induced by INF1 in *NbEAS*- or *NbEAH*-silenced plants is comparable with the control plant (Supplementary Fig. 5). These results indicated that a major consequence of activation of the ET signaling pathway in response to an attack by *P. infestans* is capsidiol production, which plays a crucial role for preventing the extension of *P. infestans* in mature *N. benthamiana* plants (Supplementary Fig. 6).

DISCUSSION

N. benthamiana has “adult resistance” against *P. infestans*.

Previously, it was not clear whether *N. benthamiana* was resistant against *P. infestans* because of contradictory reports about their interaction. (Kamoun et al. 1998; Yoshioka et al. 2003; Becktell et al. 2006; Asai et al. 2008). In this study, we revealed that mature plants of *N. benthamiana* show robust

resistance against *P. infestans*, whereas relatively young plants are always susceptible to this pathogen (Fig. 1). This age-related resistance or “adult resistance” has been found in some plant–microbe interactions (Panter and Jones 2002; Develey-Rivière and Galiana 2007). In tobacco, increased resistance against *P. parasitica* and TMV has been observed in the flowering stage (Yalpani et al. 1991; Hugot et al. 1999). In *Arabidopsis*, age-related resistance to bacterial pathogen *Pseudomonas syringae* pv. *tomato* and oomycete pathogen *H. arabidopsidis* correlated with the transition to flowering. However, the same resistance was not effective for necrotrophic bacterial (*Erwinia carotovora*) and fungal (*Botrytis cinerea*) pathogens and biotrophic fungal pathogen *Erysiphe cichoracearum* (Rusterucci et al. 2005).

McDowell and associates (2005) reported that *H. arabidopsidis* isolate Emco5 displayed full virulence on cotyledons of *Arabidopsis* ecotype Col-0 but induced weak resistance reactions in true leaves, which were conditional on a single locus

Fig. 6. Expression of capsidiol biosynthesis genes, *NbEAS* and *NbEAH*, is regulated by ethylene and is essential for the resistance of *Nicotiana benthamiana* against *Phytophthora infestans*. *EAS* = 5-*epi*-aristolochene synthase and *EAH* = 5-*epi*-aristolochene dihydroxylase. **A**, *N. benthamiana* plants were incubated with ethylene at 100 ppm for 6 h and expression of *NbEAS* and *NbEAH* was detected by reverse-transcription polymerase chain reaction (RT-PCR) using gene-specific primers. Control plants (mock) were incubated under identical conditions in the absence of added ethylene. **B**, *N. benthamiana* was inoculated with *Tobacco rattle virus* (TRV), TRV:EAS, or TRV:EAH and total RNA was isolated from *N. benthamiana* leaves 12 h after treatment with 150 nM INF1. Expression of *NbEAS* and *NbEAH* were detected by RT-PCR using gene-specific primers. **C**, Leaves of *NbEAS*- and *NbEAH*-silenced *N. benthamiana* were inoculated with *P. infestans* and photographs were taken 9 days after inoculation. Arrowheads indicate the point of inoculation. **D**, Microscopic observation of *N. benthamiana* leaves 48 h after inoculation with *P. infestans*. Inoculated leaves were stained with lactophenol trypan blue to visualize dead plant cells and the hyphae of *P. infestans*. Arrowheads indicate the hyphae of *P. infestans*. Bars = 50 μm. Similar results were obtained from at least 12 inoculated leaves. **E**, Plots showing the severity of disease symptoms as percentages in each of five classes of *N. benthamiana*. Leaves of *NbEAS*-, *NbEAH*-, or *NbEIN2*-silenced or control plants were inoculated with *P. infestans* and severities of disease symptom were evaluated from 1 to 5 days after inoculation. At least 12 inoculated leaves from each silenced plant were counted.

RPP31. In interactions between tomato and leaf mold pathogen *Cladosporium fulvum*, both tomato *Cf-9* and *Cf-9B* conferred resistance to *C. fulvum* with an avirulence gene in mature plants, whereas only *Cf-9* was effective for seedling resistance (Panter et al. 2002). In rice, *Xa21*-dependent resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* progressively increases from the susceptible two-leaf stage through to the resistant nine- to ten-leaf stage (Century et al. 1999). These reports indicated that, at least in some interactions, mechanisms similar to R protein-mediated recognition of pathogens is involved in age-related resistance. In interactions between *N. benthamiana* and *Phytophthora infestans*, the primary candidate for the *P. infestans* avirulence factor is secretory protein INF1, because a previous report indicated that *P. infestans* with suppressed expression of the *inf1* gene became more pathogenic to *N. benthamiana* (Kamoun et al. 1998). However, another report indicated that expression of the *inf1* gene was subtle in the biotrophic phase of infection, probably to avoid the recognition by the host plant, but then upregulated in the necrotrophic phase of infection, most likely for uptake of sterol from the plant plasma membrane (Kamoun et al. 1997; Blein et al. 2002). Therefore, we cannot rule out the possibility that a factor other than INF1 is the primary factor in the induction of age-related resistance of *N. benthamiana* to *P. infestans*.

Penetration resistance would be a part of *N. benthamiana* resistance against *P. infestans*.

The *Arabidopsis pen2* mutant has impaired penetration resistance against nonpathogenic powdery mildew *Blumeria graminis* f. sp. *hordei*. This mutant also showed reduced penetration resistance against *P. infestans* with increased incidence of hypersensitive cell death, indicating that penetration resistance is backed up by an HR-dependent defense mechanism (Lipka et al. 2005). The *pen2* mutant, unlike the wild-type plant, developed visible necrosis after inoculation with *P. infestans*, and trypan blue staining revealed an increased number of dead cells (Westphal et al. 2008). In interactions between mature *N. benthamiana* and *P. infestans*, microscopic observation with trypan blue staining revealed the induction of a few HR in epidermal and mesophyll cells of *N. benthamiana* but infection by the majority of the zoospores inoculated ceased at the surface of plant leaves without induction of cell death. This is in contrast to the macroscopically visible necrotic lesions on potato leaves inoculated with avirulent *P. infestans* (Kitazawa and Tomiyama 1969; Ballvora et al. 2002). Our observations indicate that penetration resistance is a basal resistance mechanism of *N. benthamiana* against *P. infestans*.

SA signaling but not NPR1 is involved in age-related resistance of *N. benthamiana* against *P. infestans*.

Several previous reports indicated that age-related resistance in various but not all plant-pathogen interactions is dependent on production of SA (Develey-Rivière and Galiana 2007). In this study, young and susceptible *N. benthamiana* plants showed resistance to *P. infestans* after pretreatment with a functional analog of SA, ASM. This suggested that a lack of resistance in young *N. benthamiana* is a result of failure in induction of the SA-mediated signaling pathway. Moreover, silencing of the *NbICS1* gene compromised resistance of mature plants against *P. infestans*, confirming the importance of SA signaling in age-related resistance of *N. benthamiana* against *P. infestans*.

NPR1 is a central factor of SA signaling in disease resistance, which regulates the expression of various SA-regulated genes, including a marker gene for induction of disease resis-

tance, *PR-1a* (Shah 2003; Pieterse and van Loon 2004). Although *NbNPR1* was reported as a gene required for *N*-gene-dependent resistance against TMV in *N. benthamiana* (Liu et al. 2002), this gene was not required for age-related resistance against *P. infestans* (Fig. 2). Two genes regulated by SA signaling, *NbPR-1a* and *NbVPE*, were not required for the resistance of *N. benthamiana* against *P. infestans*. Rivière and associates (2008) reported that silencing of the *PR1* gene in *N. tabacum* had no impact on age-related resistance against *P. parasitica*, which is consistent with our result. *NbVPE* was reported to be required for the induction of *N*-gene-dependent HR (Hatsugai et al. 2004). These previous reports and our data indicate that, although resistance against both *P. infestans* and TMV requires activation of a SA signaling pathway, there are different sets of SA-regulated mechanisms directly involved in resistance against these pathogens.

SA synthesis regulated by ICS1 but not by PAL is essential for the resistance of *N. benthamiana* against *P. infestans*.

There are two known pathways for SA biosynthesis in plants. SA synthesis from phenylalanine via trans-cinnamic acid and benzoic acid has been reported in various plant species, including tobacco and *Arabidopsis* (Mauch-Mani and Slusarenko 1996; Ribnicky et al. 1998). Relatively recently, an alternative pathway for SA production from chorismate via isochorismate was found in plants (Wildermuth et al. 2001). This pathway was originally identified in bacteria (Serino et al. 1995). In interactions between TMV and tobacco, PAL and benzoic acid 2-hydroxylase (BA2H) activity is induced by inoculation with the pathogen (León et al. 1993; Bate et al. 1994). Moreover, accumulation of SA was reduced in transgenic tobacco with suppressed expression of the *PAL* gene (Pallas et al. 1996). These reports suggest that SA is primarily synthesized via the pathway involving PAL and BA2H for TMV resistance in tobacco. However, there is no report in *Arabidopsis* about the involvement of PAL in disease resistance despite screening extensively for mutants which show reduced disease resistance with various pathogens (Thatcher et al. 2005). Therefore, it is most likely that PAL is not a major factor in disease resistance in *Arabidopsis*. In contrast, *Arabidopsis* mutant *sid2* has a disrupted *ICS1* gene and shows reduced resistance to various pathogens, including *H. arabidopsidis* and *Pseudomonas syringae*. This indicates that *ICS1*-mediated SA biosynthesis is central to the disease resistance of *Arabidopsis* against some pathogens (Glazebrook 2005; Vlot et al. 2009). For SA biosynthesis in response to ozone (O₃) exposure, SA is produced by a PAL-mediated pathway in *N. tabacum*, whereas *Arabidopsis* produces SA via isochorismate (Ogawa et al. 2005). This report indicated that the pathway for SA production is variable between species, even during a response to the same type of stress. In this study, we revealed that *NbICS1* but not *NbPAL* is essential for the resistance of *N. benthamiana* against *Phytophthora infestans* (Fig. 2). Although expression of *NbPAL* is induced by INF1 treatment, gene silencing of *NbPAL* had no effect on resistance of *N. benthamiana* against *P. infestans*, suggesting that the *ICS1*-mediated pathway is the central biosynthetic pathway for SA in disease resistance of *N. benthamiana*. Consistently, Catinot and associates (2008) have reported that production of SA, induced by inoculation with a bacterial pathogen as well as exposure to UV light, is suppressed in *NbICS1*-silenced plants.

Silencing of *ICS1* enhanced cell death induced by INF1 and NPP1.1.

NbICS1-silenced plants showed accelerated cell death by apoplastic expression of either INF1 or NPP1.1 (Fig. 3). It was reported that cell death induced by NPP1.1 is dependent on

COI1, *MEK2*, *NPR1*, and *TGA2.2* but silencing of these genes had no influence on cell death induced by INF1 (Kanneganti et al. 2006). Therefore, the extensive cell death response in *NbICS1*-silenced plants is probably the result of unsuccessful termination of cell death induced in neighboring cells. It is not clear whether this accelerated cell death is a cause of enhanced susceptibility in mature *N. benthamiana*; however, it is expected that accelerated cell death might facilitate the growth of *P. infestans* in the necrotrophic phase.

The ET-mediated signaling pathway regulates the production of capsidiol in *N. benthamiana*.

In this study, it was shown that ET is essential and sufficient to induce the expression of *NbEAS* (Figs. 5 and 6). Consistent with our data, a recent report by Maldonado-Bonilla and associates (2008) indicated that the promoter region of the *EAS* gene from chili pepper (*Capsicum annuum*) and tobacco (*N. tabacum*) have an ET-responsive element (GCCGCC). Given that expression of *NbEAS* is also regulated by ET, then the production of capsidiol in *N. benthamiana* is primarily regulated by the ET-mediated signal transduction pathway. Previous circumstantial evidence also indicated a possible link between production of ET and capsidiol in tobacco suspension cells treated with the elicitor protein cryptogin from *P. cryptogea* (Milat et al. 1991). Our report shows the first clear evidence indicating that production of capsidiol in *Nicotiana* spp. is directly regulated by ET and involved in disease resistance. Phytoalexins from different plant species are chemically diverse, including terpenoids, glycosteroids, and alkaloids, and the phytoalexins are produced by distinctive pathways (Hammerschmidt 1999). Indolic alkaloid phytoalexin of *Arabidopsis*, camalexin, does not contribute to resistance to the biotrophic oomycete *H. arabidopsidis* (Glazebrook et al. 1997) and fungus *E. orontii* (Reuber et al. 1998), whereas this phytoalexin is essential for resistance against the necrotrophic fungal pathogens *Alternaria brassicicola* (Thomma et al. 1999) and *Botrytis cinerea* (Ferrari et al. 2003). ET signaling is not substantially involved in production of camalexin in *Arabidopsis* because mutation in *ein2* did not compromise resistance against *A. brassicicola* and *B. cinerea* (Thomma et al. 1999; Ferrari et al. 2003).

Regulation and role of ROS production in the interaction between *N. benthamiana* and *P. infestans*.

Rapid production of ROS is one of the earliest reactions during the induction of plant disease resistance (Doke et al. 1996; Torres and Dangle 2005). Rbohs from different plant species have been identified as essential enzymes for the induction of ROS in disease resistance (Simon-Plas et al. 2002; Torres et al. 2002; Yoshioka et al. 2003). Production of ROS in an *Arabidopsis rbohD/rbohF* double mutant was significantly reduced in response to inoculation with avirulent bacterial and oomycete pathogens (Torres et al. 2002). Similarly, VIGS of *NbRbohA* and *NbRbohB*, orthologs of *Arabidopsis rbohD* and *rbohF*, in *N. benthamiana* compromised ROS accumulation during a *P. infestans* infection (Yoshioka et al. 2003). Lherminier and associates (2009) reported that *N. tabacum* transformed with antisense *NtRbohD* showed reduced ROS production in response to the elicitor protein, cryptogin. In this study, significant reduction of elicitor-induced ROS production in *NbICS1*- and *NbEIN2*-silenced *N. benthamiana* was observed (Fig. 4). Given the expression level of the gene for *NbRbohB*, a NADPH oxidase for ROS production in disease resistance (Asai et al. 2008), is not altered when these silenced plants are treated with INF1 (Fig. 5), and regulation of ROS production by either SA or ET is probably posttranscriptional. There are some reports showing that application of SA can promote ROS production, whereas others show that ROS production could enhance SA

accumulation (Overmyer et al. 2003). Based on these reports, it is proposed that production of SA and ROS form a self-amplifying loop. There are relatively few reports about regulation of ROS production by ET. In rice roots, adventitious root primordia are formed at the nodes of the stem during plant development, and root growth is preceded by death of epidermal cells that cover the root primordial. Emergence of the root is promoted by ET-induced ROS production followed by local death of epidermal cells, indicating that ET acts upstream of ROS in epidermal cell-death signaling (Steffens and Sauter 2009). In the interaction between *Arabidopsis* and *A. brassicicola*, application of aminoethoxyvinylglycine, an inhibitor of ET biosynthesis, reduced the RBOHD-dependent ROS generation (Pogány et al. 2009).

Although it is clear that particular isoforms of Rboh are involved in ROS production in plant disease resistance, knockout or knockdown of *Rboh* genes have contrasting effects in different plant-microbe interactions. *Arabidopsis rbohD/rbohF* mutants displayed reduced HR in response to inoculation with avirulent *Pseudomonas syringae* pv. *tomato* compared with the wild type but bacterial growth in the host plant was not affected (Torres et al. 2002). Moreover, in adult resistance of *Arabidopsis* Col-0 against *H. arabidopsidis* isolate Emco 5, *rbohD/rbohF* double mutants displayed enhanced induction of cell death and resistance (Torres et al. 2002). Pogány and associates (2009) reported that *Arabidopsis* RbohD has dual roles in cell-death induction in the *Arabidopsis-Alternaria* pathosystem. In this system, ROS produced by RbohD promotes cell death in the first cell directly attacked by the pathogen while also suppressing the spread of cell death to neighboring cells. Knockout of *rbohD*, however, had no impact on the infection of *A. brassicicola*. *N. tabacum* with antisense *NtRbohD* showed comparable resistance to the wild-type plant against *Phytophthora parasitica* (Lherminier et al. 2009). In contrast, Yoshioka and associates (2003) indicated that *Potato virus X*-mediated VIGS of either *NbRbohA* or *NbRbohB* partially compromised resistance of *N. benthamiana* to *P. infestans*. However, in our experimental conditions, TRV-mediated VIGS of *NbRbohB* had no obvious effect on the resistance of mature *N. benthamiana* to *P. infestans*, although we detected almost complete loss of ROS production induced by INF1 in the *NbRbohB*-silenced plants (Y. Shibata and D. Takemoto, unpublished data). Therefore, although gene silencing of *NbICS1* and *NbEIN2* compromised both disease resistance and ROS production, we consider that ROS is unlikely to be directly involved in age-related resistance of *N. benthamiana* to *P. infestans*.

In summary, our data indicate that age-related resistance of *N. benthamiana* to *P. infestans* requires activation of both SA- and ET-mediated signal transduction pathways. Microscopic observation revealed that penetration resistance is partially effective for *N. benthamiana* resistance against *P. infestans*. Although knockdown of either SA- or ET-mediated signaling compromised ROS production induced by an elicitor, we consider that production of ROS is not directly involved in disease resistance. We propose that *N. benthamiana* requires SA-regulated appropriate induction of hypersensitive cell death and ET-regulated production of phytoalexin to prevent infection during both the biotrophic and necrotrophic phases of *P. infestans*.

MATERIALS AND METHODS

Biological materials, growth conditions, and inoculation.

N. benthamiana plants were grown in an environmentally controlled growth room at 23°C with 16 h of light per day. *P. infestans* isolates were either maintained on susceptible potato (*S. tuberosum*) tubers (isolates PI0-1 and PI1234-1) or on rye media (isolates NE0805, IG0803, MR0803, 08YB1, 08YD1,

and 08MB), at 20°C. Collection of zoosporangia and induction of zoospore production from *P. infestans* PI0-1 or PI1234-1 was performed as described by Doke (1975). Zoosporangia suspensions from the remaining *P. infestans* isolates were prepared as follows. *P. infestans* isolates were subcultured on rye media for 7 to 10 days and 20 ml of water was added to the surface of the *P. infestans* colonies, which were then rubbed with a cotton swab to release the zoosporangia. Induction of zoospores was performed as described previously (Doke 1975). Leaves of *N. benthamiana* were inoculated with a 1-ml aliquot of *P. infestans* zoospores (2×10^5 zoospores/ml) and covered with lens paper to keep the suspension of zoospores on the surface of the leaves. The inoculated plants were kept at high humidity at 20°C for 1 day after the inoculation, then were moved to a growth room at 23°C.

Treatment of *N. benthamiana* with chemicals.

Treatment with ASM was performed as follows. ASM (benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester) (Vallad and Goodman 2004) at 100 ppm with 10% (wt/vol) wettable powder was treated by soil drenching (approximately 40 ml per 300 ml of soil) 2 days before the inoculation of *P. infestans*. Control plants were treated with same amount of water with 10% (wt/vol) soluble powder. Treatment with ET was performed as follows. *N. benthamiana* plants (35 days old) were placed in a gas-tight acryl chamber. ET was introduced into the chamber at a final concentration of 100 ppm and plants were incubated at 23°C for 6 h. Control plants were incubated under identical conditions in the absence of added ET.

Preparation and treatment of INF1 elicitor.

Escherichia coli cells (DH5 α) carrying a chimeric plasmid containing *inf1*, pFB53 (Kamoun et al. 1997), were cultured overnight at 37°C, diluted (1:100) in Luria-Bertani (LB) medium with ampicillin at 50 μ g/ml, and grown until the optical density at 600 nm (OD₆₀₀) of the culture reached 0.6. Production of INF1 was induced by adding 0.4 mM isopropyl β -D-thiogalactopyranoside for 3 to 4 h. The culture was centrifuged and the resultant supernatant was dialyzed against water with SnakeSkin dialysis tubing (7-kDa molecular mass cutoff; Pierce Biotechnology, Rockford, IL, U.S.A.) overnight at 4°C.

DNA sequencing and bioinformatics.

DNA sequencing utilized the dideoxynucleotide chain termination method using Big-Dye (version 3) chemistry (Applied Biosystems, Foster City, CA, U.S.A.) and the separation of products on an ABI3130 analyzer (Applied Biosystems). Sequence data was analyzed and annotated in MacVector (version 10.6; MacVector Inc., Cary, NC, U.S.A.) or GENETYX (version 7; GENETYX Corp., Tokyo). Sequences of cDNA for *NbICS1* (Catinot et al. 2008) (accession no. EU257505), *NbCOII* (Liu et al. 2004) (AY547493), and *NbVPE1-a* (Hatsugai et al. 2004) (AB181187) have been published. The sequence of cDNA for *NbEAS* was obtained from the *N. benthamiana* expressed sequence tag (EST) database (PlantGDB, Sequence ID; PUT-173a-Nicotiana_benthamiana-17394) by searching with the sequence of *N. tabacum* *EAS* (Wu et al. 2005) (AY313939). The sequence of cDNA for *NbEIN2* was obtained from the EST database (PUT-173a-Nicotiana_benthamiana-110727) by searching with the sequence of *Arabidopsis thaliana* *EIN2* (Alonso et al. 1999) (AAD41076). Partial cDNAs for *NbNPR1*, *NbPAL*, *NbPR1a*, *Nbhsr203j*, and *NbEAH* were amplified from *N. benthamiana* cDNA with specific primers designed from *N. tabacum* sequences for *NPR1* (Liu et al. 2002) (AF480488), *PAL1* (Nagai et al. 1994) (D17467), *PR1a* (Cutt et al. 1988) (X12485), *hsr203j* (Pontier et al. 1998) (X77136), and *EAH* (Ralston et al. 2001) (AF368376).

RT-PCR.

Total RNAs were isolated from *N. benthamiana* leaves using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's recommendations. cDNA synthesis was conducted using a commercial kit (ReverTra Ace- α -; Toyobo, Osaka, Japan) and PCR was performed with GoTaq Master Mix (Promega Corp., Madison, WI, U.S.A.). The thermocycle conditions used were one cycle at 94°C for 2 min; and *x* cycles of 94°C for 30 s, 55°C for 30 s (50°C for *NbEIN2*), 72°C for 1 min (per kilobase), and 72°C for 5 min. Gene-specific primer (Supplementary Table 1) combinations used and the number of PCR cycles for expression analysis were as follows: ICS1-F-H and GE-ICS1-R (*NbICS1*), 25 cycles; GE-EIN2-F and GE-EIN2-R (*NbEIN2*), 32 cycles; NPR1-F-H and GE-NPR1-R (*NbNPR1*), 25 cycles; COI1-F-C and GE-COI1-R (*NbCOII*), 25 cycles; GE-VPE-F and VPE-R-B (*NbVPE*), 25 cycles; GE-PR-1a-F and PR-1a-R (*NbPR-1a*), 25 cycles; GE-HSR203J-F and GE-HSR203J-R (*Nbhsr203j*), 25 cycles; EAS-F-H and GE-EAS-R (*NbEAS*), 18 cycles; EAH-F and GE-EAH-R (*NbEAH*), 20 cycles; PAL-F and GE-PAL-R (*NbPAL*), 25 cycles; and EF1 α -F and EF1 α -R (*NbEF-1 α*), 23 cycles. The PCR products were separated on a 1.8% agarose gel and visualized under UV illumination after ethidium bromide staining.

Preparation of silencing constructs.

Partial cDNA of *NbPAL*, *NbVPE*, *NbPR-1a*, *NbEAS*, and *NbEAH* was amplified from *N. benthamiana* cDNA using gene-specific primers with restriction sites at the 5' ends. The gene-specific primer combinations used to construct the silencing vectors were as follows: VPE-F-C and VPE-R-B (*NbVPE*), PAL-F and PAL-R (*NbPAL*), PR-1a-F and PR-1a-R (*NbPR-1a*), EAS-F-H and EAS-R-B (*NbEAS*), and EAH-F and EAH-R (*NbEAH*). Amplified fragments were digested with restriction enzymes and ligated into the pTV00 vector (Ratcliff et al. 2001) digested with compatible restriction enzymes to generate pTV00:PAL, pTV00:VPE, pTV00:PR-1a, pTV00:EAS, and pTV00:EAH. For all constructs, the inserts were amplified and sequenced to confirm cloning of the correct gene fragment. Construction of silencing vectors for *NbICS1*, *NbNPR1*, *NbCOII*, and *NbEIN2* has been reported previously (Uruma et al. 2009). The pTV00 vectors were transformed into *Agrobacterium tumefaciens* (strain GV3101, with helper plasmid pSoup) by electroporation with a MicroPulser electroporator (Bio-Rad, Hercules, CA, U.S.A.) and transformants were selected on LB media with rifampicin at 50 μ g/ml, kanamycin at 50 μ g/ml, and tetracycline at 2.5 μ g/ml.

VIGS.

Induction of VIGS was performed as previously reported (Ratcliff et al. 2001). *A. tumefaciens* GV3101, carrying the binary TRV RNA 1 construct pBINTRA6 and the TRV RNA2 vector pTV00 or pTV00 derivatives, was cultured to saturation in LB medium. Bacterial suspensions were then collected by centrifugation at 16,000 $\times$ g for 1 min. The bacterial cells were then resuspended in 10 mM morpholineethane sulfonic acid (MES)-NaOH (pH 5.6), 10 mM MgCl₂, and 150 μ M acetosyringone (final OD₆₀₀ = 0.5) and incubated at room temperature for 2 h. The cultures were mixed in a 1:1 ratio (RNA1/RNA2) to infiltrate leaves of *N. benthamiana* using a syringe without a needle. Three to four weeks after infiltration, the upper leaves of the inoculated plants were used for experiments.

Construction of vectors for agroinfiltration.

Genes for mature *P. infestans* INF1 or NPP1.1 (without the N-terminal signal peptide) were amplified from genomic DNA of *P. infestans* PI0-1 with primers INF1-F-B and INF1-R-Sp or NPP1-F-B and NPP1-R-Sp, respectively. The signal peptides

for tobacco PR1a, *Xba*I-SpPR1-*Bam*HI, from pCBJ-sp-capsicine (Takemoto et al. 2005), *Bam*HI-INF1-*Sph*I (or *Bam*HI-NPP1.1-*Sph*I), and *Sph*I-HA/Str2-*Eco*RI (hemagglutinin tag YPYDVPDYA [Kolodziej and Young 1991] followed by a Strep tag II WSHPQFEK, [Schmidt and Skerra 1993]) were subsequently cloned into the *Xba*I-*Eco*RI site of pEI2 Ω vector (Ohtsubo et al. 1999) to generate pEI2 Ω -Sp-INF1 (or pEI2 Ω -Sp-NPP1.1). The pEI2 Ω vectors were transformed into *A. tumefaciens* (rifampicin-resistant strain C58C1) by electroporation with a MicroPulser electroporator (Bio-Rad). *A. tumefaciens* transformants were selected on LB media with rifampicin at 50 μ g/ml, kanamycin at 50 μ g/ml, and ampicillin at 50 μ g/ml.

Transient gene expression by agroinfiltration.

A. tumefaciens carrying the pEI2 Ω construct was cultured to saturation in LB medium and bacterial cells were collected by centrifugation at 16,000 $\times$ g for 1 min. The bacterial cells were then resuspended in MMA infiltration medium (MS salts at 5 mg/ml, MES at 1.95 mg/ml, sucrose at 20 mg/ml, and 200 μ M acetosyringone, pH = 5.6) to a final OD₆₀₀ of 0.5 and incubated at room temperature for 2 h. The cultures were allowed to infiltrate the intercellular space of *N. benthamiana* leaves using a syringe without a needle.

Protein extraction and immunoblotting.

N. benthamiana leaf tissue was harvested at 48 h after inoculation with *A. tumefaciens* and stored at -80°C after fixing in liquid nitrogen. Protein extracts from *N. benthamiana* leaves were prepared by grinding them in a buffer consisting of: 50 mM MOPS, 0.5 M sorbitol, 5 mM EDTA, 5 mM EGTA, 100 mM NaF, and 100 μ M Na₂VO₄, pH 7.6, and containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis,). Supernatants were cleared by centrifugation at 16,000 $\times$ g for 10 min at 4°C and stored in a -80°C freezer. Protein concentration was determined using the Protein Assay (Bio-Rad) with bovine serum albumin as a standard. HA-tagged INF1 and NPP1.1 expressed in *N. benthamiana* leaves were detected by immunoblotting performed as described by Kato and associates (2008) using monoclonal HA antibodies (Roche) and anti-mouse immunoglobulin G antibody (GE Healthcare).

ROS measurement.

The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence. L-012 (Wako, Osaka, Japan) is a luminol derivative that is highly sensitive to superoxide anion (O₂⁻) (Asai et al. 2008; Nishinaka et al. 1993). To detect the ROS production in *N. benthamiana* leaves, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) was allowed to infiltrate to the intercellular space of leaves via a syringe without needle. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in a dark chamber at 20°C (Aquacosmos 2.5; Hamamatsu Photonics, Shizuoka, Japan), and quantified using the U7501 program (Hamamatsu Photonics).

Microscopy.

To visualize plant cell death and colonization of *P. infestans*, leaves of *N. benthamiana* were stained with lactophenol trypan blue as described by Takemoto and associates (2003) with a minor modification. Briefly, infected leaves were cleared in methanol overnight, and then the cleared tissue was boiled for 2 min in lactophenol trypan blue stain (10 ml of H₂O, 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue). After the leaves had been allowed to cool at room temperature for 1 h, the stain was replaced with chloral hydrate at 1 g/ml. Stained leaves were monitored using an Olympus microscope BX51 (Olympus, Tokyo).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Plant genome database (PlantGDB) website: plantgdb.org