Nicotiana benthamiana* Calreticulin 3a Is Required for the Ethylene-Mediated Production of Phytoalexins and Disease Resistance Against Oomycete Pathogen *Phytophthora infestans

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Mature *Nicotiana benthamiana* shows strong resistance to the potato late blight pathogen *Phytophthora infestans*. By screening using virus-induced random gene silencing, we isolated a gene for plant-specific calreticulin *NbCRT3a* as a required gene for resistance of *N. benthamiana* against *P. infestans*. *NbCRT3a* encodes an endoplasmic reticulum quality-control (ERQC) chaperone for the maturation of glycoproteins, including glycosylated cell-surface receptors. *NbCRT3a*-silenced plants showed no detectable growth defects but resistance to *P. infestans* was significantly compromised. Defense responses induced by the treatment with INF1 (a secretory protein of *P. infestans*), such as production of reactive oxygen species and accumulation of phytoalexins, were suppressed in *NbCRT3a*-silenced *N. benthamiana*. Expression of an ethylene-regulated gene for phytoalexin biosynthesis, *NbEAS*, was reduced in *NbCRT3a*-silenced plants, whereas the expression of salicylic acid-regulated *NbPR-1a* was not affected. Consistently, induction of ethylene production by INF1 was suppressed in *NbCRT3a*-silenced plants. Resistance reactions induced by a hyphal wall components elicitor prepared from *P. infestans* were also impaired in *NbCRT3a*-silenced plants. However, cell death induced by active mitogen-activated protein kinase kinase (*NbMEK2^{DP}*) was not affected by the silencing of *NbCRT3a*. Thus, *NbCRT3a* is required for the initiation of resistance reactions of *N. benthamiana* in response to elicitor molecules derived from *P. infestans*.

Phytophthora infestans is the causal agent of potato late blight, one of the most devastating and economically significant plant diseases. This pathogen is also historically momentous as a causal agent of the Irish potato famine in the 1840s. Even now, *P. infestans* costs more than \$3 billion per year worldwide for control treatments as well as yield losses (Fry 2008). Generally, the interactions between potato cultivars and *P. infestans* races are determined by the combination of race-

specific resistance (R) proteins of the plant and avirulence (Avr) factors (or effectors) of the pathogen, which is recognized by corresponding R proteins. There are approximately 10 dominant R genes that have been identified in the wild potato species *Solanum demissum*, and some of these were introduced into cultivated potato varieties. However, R-gene-based resistances were eventually defeated by emerging *P. infestans* strains which lost corresponding effector genes (Fry 2008; Umaerus and Umaerus 1994). R genes for broad-spectrum resistance to *P. infestans* have been identified, including *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005). Because these broad-spectrum R proteins are involved in the recognition of the widely distributed effector proteins among *P. infestans* strains (*ipiO* for *Rpi-blb1* and *Avrblb2* for *Rpi-blb2*) (Champouret et al. 2009; Oh et al. 2009), resistance dependent on broad-spectrum R proteins could be defeated, as in the case of the resistance by race-specific R proteins (Champouret et al. 2009).

Recognition of general molecules derived from microorganisms is an essential process for plant cells to induce effective disease resistance against potential pathogens. Structural molecules such as fungal cell wall constituents (chitin, glucans, fatty acids, proteins, and glycoproteins), bacterial lipopolysaccharide, and flagellin elicit defense responses in a wide range of plant species. Such nonspecific elicitors are called microorganism- or pathogen-associated molecular patterns (MAMPs or PAMPs), which are recognized by plant receptors to induce innate immunity of plants (Jones and Takemoto 2004). *P. infestans* produces a large number of secretory proteins during the infection process, including apoplastic proteins such as INF1 and NPP1.1 (Fellbrich et al. 2002; Kamoun et al. 1997) and intracellular effectors (Armstrong et al. 2005; Vleeshouwers et al. 2008). Elicitins, the major secretory proteins produced by *Phytophthora* and *Pythium* spp., are sterol-carrier proteins that enable the pathogen to uptake sterols from plasma membranes of host plants (Blein et al. 2002). Elicitins are considered as semi-PAMPs of oomycete pathogens because they are conserved only among two genera, and act as elicitors for a limited range of dicot plants, including *Nicotiana*, *Brassica*, and *Solanum* spp. (Kamoun et al. 1993; Takemoto et al. 2005; Vleeshouwers et al. 2006). Fatty acids such as eicosapentaenoic acid and arachidonic acid from mycelia of *P. infestans* were identified as active elicitors of phytoalexin production in potato (Bostock et al. 1981). β -1,6-1,3 heptaglucan extracted from *P. sojae* also showed elicitor activity in soybean and

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tobacco (Brady et al. 1993; Sharp et al. 1984). Such molecules derived from mycelial walls of *Phytophthora* spp. could act as MAMPs of oomycete pathogens.

Although *P. infestans* is a practically important pathogen, genes required for plant resistance against *P. infestans* are largely unknown except for *R* genes, perhaps because potato, the natural host of *P. infestans*, is not the ideal material for molecular-biology-based studies. In recent years, *Nicotiana benthamiana* has been widely used as a Solanaceae model plant, because there are established methods for functional analysis of genes by *Agrobacterium*-mediated transient gene expression or virus-induced gene silencing (VIGS) (Goodin et al. 2008; Ratcliff et al. 2001). We previously showed that mature *N. benthamiana* plants are resistant to eight isolates of *P. infestans*, whereas relatively young plants were susceptible to all of the tested isolates, indicating that *N. benthamiana* has age-dependent nonhost resistance against *P. infestans* (Shibata et al. 2011). Therefore, *N. benthamiana* can be used as a model host plant to investigate plant genes for the resistance against *P. infestans*.

We have identified several genes required for the resistance of mature *N. benthamiana* plants to *P. infestans*. Analysis with VIGS revealed that both NbICS1 and NbEIN2, the genes for salicylic acid (SA) biosynthesis and ethylene signaling, respectively, are required for the resistance of *N. benthamiana* against *P. infestans*. Silencing of genes for phytoalexin (capsidiol) biosynthesis, 5-*epi*-aristrochen synthase (*NbEAS*), and 5-*epi*-aristrochen dihydroxylase (*NbEAH*) compromised resistance of *N. benthamiana* to *P. infestans*. Expression of *NbEAS* and *NbEAH* is induced by the treatment of ethylene and suppressed by silencing of *NbEIN2*, indicating that production of phytoalexin is regulated by ethylene in *N. benthamiana* (Shibata et al. 2010).

In the present study, we developed a VIGS-based screening system to isolate new genes required for the nonhost resistance of *N. benthamiana* against *P. infestans*. In this manuscript, we have particularly focused on the role of an isolated gene encoding Calreticulin 3a, NbCRT3a, on the induction of various resistance reactions in response to elicitors derived from *P. infestans*.

RESULTS

Construction of a screening system for the isolation of genes required for the resistance of *N. benthamiana* against *P. infestans*.

Mature *N. benthamiana* plants (>35 days old) show strong resistance to *P. infestans* (Shibata et al. 2011). By using VIGS of targeted candidate genes, we have identified several genes required for the resistance of *N. benthamiana* against *P. infestans*, such as *NbEAS*, *NbICS1*, *NbEIN2*, *NbSGT1*, and *NbHSP90* (Shibata et al. 2010, 2011). To further identify genes required for the resistance of *N. benthamiana*, we randomly cloned cDNA fragments of *N. benthamiana* in the vector pTV00 for VIGS (Ratcliff et al. 2001). Leaves of *N. benthamiana* treated with 150 nM INF1, a secreted elicitor protein of *P. infestans* (Kamoun et al. 1997), were harvested 1, 3, 6, 12, and 24 h after the treatment. By suppressive subtractive hybridization using control cDNA from untreated *N. benthamiana* leaves, cDNA of genes induced by INF1 treatment was enriched and equalized. *Rsa*I-digested cDNA was ligated with linkers, amplified by polymerase chain reaction (PCR) with primers with *Bam*HI or *Hind*III, and cloned into *Bam*HI-*Hind*III sites of pTV00. The average size of integrated fragments was approximately 700 bp (data not shown).

The constructed cDNA library in pTV00 was transformed to *Agrobacterium tumefaciens* GV3101 and used for induction of

VIGS. Approximately 2,000 plants have been inoculated with *A. tumefaciens* harboring individual VIGS vector. Approximately 17% of plants showed some kind of growth defect such as dwarfing, chlorosis, or spontaneous cell death (data not shown). By using plants infected with *Tobacco rattle virus* (TRV) and TRV:EAS as resistant and susceptible control plants, respectively, mature *N. benthamiana* plants with random silencing of genes were inoculated with *P. infestans* isolate 08YD1 (Shibata et al. 2011). Among 2,000 plants, 63 showed development of disease symptoms within 10 days after the inoculation of *P. infestans*. Twenty-six unique genes have been isolated from 63 plant lines as candidate genes required for the resistance against *P. infestans*. In this manuscript, we will present a detailed analysis of candidate lines A3-199 and A2-30. Further studies on other lines are in progress and will be reported elsewhere.

Identification of *NbCRT* genes from *N. benthamiana*.

By primary screening of *N. benthamiana* lines showing reduced resistance against *P. infestans*, two candidate lines—A3-199 with weak disease symptom and A2-30 with significant development of disease symptoms—were isolated (Fig. 1A). Vectors used for the induction of VIGS for A3-199 and A2-30 lines were isolated from *A. tumefaciens* and integrated cDNA fragments were sequenced. By analysis of the draft genome sequence of *N. benthamiana* (ver. 0.4.2; Sol Genomics Network) (Bombarely et al. 2012), full coding sequences of these cDNAs were estimated. The cDNA of coding sequences was amplified by reverse-transcriptase (RT)-PCR to experimentally confirm the predicted sequence (Supplementary Fig. S1). The silencing vector for A3-199 contained a 342-bp cDNA fragment encoding calreticulin (CRT), an endoplasmic reticulum resident quality-control chaperone. The closest homolog of the isolated CRT gene in the *Arabidopsis* genome was *AtCRT2* (At1g09210, also known as *AtCRT1b*); therefore, the isolated gene was designated *NbCRT2a*. Further analysis of the draft genome sequence identified another calreticulin2 gene, named *NbCRT2b* (Fig. 1B and C). Because the gene fragment of *NbCRT2a* in the silencing vector showed 99% identity with the corresponding region of *NbCRT2b*, expression of both *NbCRT2a* and *NbCRT2b* were suppressed in the A3-199 line (Fig. 2A). The A3-199 line was analyzed as a CRT2-silenced plant in the following analysis. A2-30 contained a 458-bp cDNA fragment encoding the plant-specific CRT, *NbCRT3a*. Two copies of *NbCRT3a* genes (*NbCRT3a1* and *NbCRT3a2*) were identified from the draft genome sequence (Fig. 1B and C). Partial sequence of another *NbCRT3*, *NbCRT3b*, has been reported in a recent study (Liebrand et al. 2012). By analysis of the draft genome sequence, the reported sequence was identified in Scf18258 (for genome sequence ver. 0.4.2); however, the part of sequence encoding C-terminal region of plant CRT3 was missing for *NbCRT3b* (data not shown). *NbCRT3a1* and *NbCRT3a2* showed 97% identity in nucleic acid sequence; therefore, expression of both *NbCRT3a1* and *NbCRT3a2* was suppressed in the A2-30 line (Fig. 2A). The A2-30 line was used as CRT3a-silenced plants in further analysis. Because the two copies of *NbCRT2* and *NbCRT3a* were highly conserved, we could not suppress the expression of single *NbCRT2* or *NbCRT3a*. As *N. benthamiana* has an allopolyploid genome (Goodin et al. 2008), two copies of *NbCRT2* and *NbCRT3a* could be derived from different ancestral *Nicotiana* spp. Given that the cDNA fragment used for the production of *NbCRT2*- and *NbCRT3a*-silenced plants did not show any significant identity with other genes in the *N. benthamiana* draft genome including between *NbCRT2* and *NbCRT3a*, it was indicated that specific target genes were suppressed in *NbCRT2*-silenced and *NbCRT3a*-silenced plants.

Fig. 1. A, Screening of gene-silenced *Nicotiana benthamiana* with reduced resistance against *Phytophthora infestans*. *N. benthamiana* with random silencing of genes were inoculated with *P. infestans* and candidate lines A2-30 and A3-199 with reduced resistance were isolated. TRV = Tobacco rattle virus. B, Phylogenetic relationships of plant calreticulins. The tree was prepared using the neighbor-joining method (Saitou and Nei 1987). Nb: *N. benthamiana*, At: *Arabidopsis thaliana*, Os: *Oryza sativa*. Gene identifiers of *Arabidopsis* and rice sequences or scaffold ID for the source of *N. benthamiana* sequences are shown. C, Comparison of the deduced amino acid sequences of *N. benthamiana* (Nb) calreticulins (CRT) with *Arabidopsis* (At) CRT1a, -1b, and -3 (Christensen et al. 2010). N-terminal signal sequence, calreticulin family sequences (CFS), the two triplicate repeats (A1-3 and B1-3) in P domain, and endoplasmic reticulum (ER) retention signal are indicated (Gelebart et al. 2005). D, Subcellular localization of GFP-CRT2a and GFP-CRT3a2 in epidermal cells of *N. benthamiana*. Expression vectors were delivered to epidermal cells of *N. benthamiana* by particle bombardment and transformed cells were monitored by confocal laser-scanning microscopy. Bar=10 μ m.

GFP-NbCRT2a and GFP-NbCRT3a2 were expressed in epidermal cells of *N. benthamiana* by particle bombardment. Both of them showed typical reticulate structure of endoplasmic reticulum (ER), indicating that NbCRT2 and NbCRT3 are primarily localized in ER (Fig. 1D).

NbCRT3a, a plant-specific calreticulin, is required for the resistance of *N. benthamiana* to *P. infestans*.

NbCRT2- and *NbCRT3a*-silenced *N. benthamiana* plants were prepared to confirm the effect of gene silencing on disease resistance against *P. infestans*. *NbCRT2*- and *NbCRT3a*-silenced plants didn't show any detectable growth defects (Supplementary Fig. S2). Using primers specific for *NbCRT2* and *NbCRT3a*, significant suppression of target genes was confirmed (Fig. 2A). Within 2 days postinoculation with *P. infestans*, *NbCRT3a*-silenced plants started to show the develop-

Fig. 2. *NbCRT3a* but not *NbCRT2* is essential for the resistance of *Nicotiana benthamiana* to *Phytophthora infestans*. **A**, *N. benthamiana* plants were inoculated with *Tobacco rattle virus* (TRV), TRV:CRT2, or TRV:CRT3a, and total RNA was isolated from *N. benthamiana* leaves. Expression of *CRT2* and *CRT3a* genes was assessed by reverse-transcriptase polymerase chain reaction with gene-specific primers. Constitutively expressing *NbEF-1α* was used as an internal standard. **B**, Top: appearance of disease symptoms on leaves. Based on severity, disease symptoms 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; 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 top panels, for leaves of control or *NbCRT2*- or *NbCRT3a*-silenced plants inoculated with *P. infestans* from 3 to 5 days postinoculation (dpi). At least 10 inoculated leaves from each silenced plant were counted.

ment of visible disease symptoms on the inoculated area. In contrast, *NbCRT2*-silenced *N. benthamiana* plants showed resistance against *P. infestans* almost comparable with control TRV-infected plants. Resistance of *NbCRT2*- and *NbCRT3a*-silenced plants was scored by visible development of disease symptoms (Fig. 2B). Although weak development of disease symptoms was occasionally observed for *NbCRT2*-silenced plants, the frequency was similar to that of control TRV-infected plants. In contrast, *NbCRT3a*-silenced plants significantly showed the development of disease symptoms, and it was comparable with that in *NbEAS*-silenced plants (Fig. 2B).

NbCRT3a-silenced *N. benthamiana* compromised reactive oxygen species production and cell death induced by the treatment of INF1.

Leaves of *NbCRT2*- or *NbCRT3a*-silenced or control *N. benthamiana* were treated with INF1, and production of reactive oxygen species (ROS) was measured as chemiluminescence using the luminol derivative, L-012, at 12 h after treatment. *NbCRT2*-silenced *N. benthamiana* showed approximately 50% reduction of ROS production compared with control TRV-infected *N. benthamiana* plants, whereas ROS produced by *NbCRT3a*-silenced plants was only 10% of control plants (Fig. 3). It was reported that ROS production positively regulates the hypersensitive response (HR) in disease resistance (Pogány et al. 2009; Torres et al. 2002). Consistently, cell death induced

Fig. 3. Effects of gene silencing of *NbCRT2* or *NbCRT3a* on INF1-induced O₂⁻ production. **A**, Leaves of *Tobacco rattle virus* (TRV)-infected *Nicotiana benthamiana* were treated with H₂O or 150 nM INF1 (left panel) and gene-silenced *N. benthamiana* were treated with 150 nM INF1 (right panels). Production of O₂⁻ was detected as L-012-mediated chemiluminescence 12 h after INF1 treatment. Circles indicate the area of treatment. Chemiluminescence images were obtained using CCD camera. **B**, Intensities of chemiluminescence were quantified with photon image processor. Data are means ± standard error (*n* = 6).

by the treatment of INF1 was reduced in *NbCRT3a*-silenced plants, whereas silencing of *NbCRT2* had no significant effect on cell death induction (Fig. 4A).

NbMEK2^{DD}-induced cell death was not affected by the silencing of *NbCRT3a*.

Given that *Arabidopsis* CRT3 was reported to be involved in the maturation and stabilization of the PAMP receptor EFR for the recognition of the bacterial elongation factor EF-Tu (Li et al. 2009; Saijo et al. 2009), *NbCRT3a* was expected to play a role in the recognition of the elicitor derived from *P. infestans*. To investigate the effect of *NbCRT3a*-silencing on the signal transduction for HR induction, a constitutive active form of mitogen-activated protein kinase kinase (MAPKK), NbMEK2^{DD} (del Pozo et al. 2004), was employed. NbMEK2^{DD} under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter was transiently expressed in control or gene-silenced plants by agroinfiltration, and the induction of cell death was monitored 48 h after the inoculation. In contrast to reduced induction of cell death in *NbCRT3a*-silenced plants treated with INF1, transiently expressed NbMEK2^{DD} can induce a similar level of cell death in control, *NbCRT2*-, and *NbCRT3a*-silenced plants (Fig. 4B). These results indicated that *NbCRT3a* is not required for MAPKK-induced HR in *N. benthamiana*.

NbCRT3a but not NbCRT2 is essential for the production of phytoalexins in *N. benthamiana*.

Generally, *Nicotiana* and *Capsicum* spp. produce a sesquiterpenoid phytoalexin, capsidiol, as an antimicrobial compound to inhibit the growth of attacking pathogens (Bailey et al. 1975). Formation of capsidiol involves the cyclization of

farnesyl pyrophosphate 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 or CYP71D21) (Ralston et al. 2001) (Fig. 5A). To our knowledge, capsidiol and related phytoalexins have not been detected in *N. benthamiana*; thus, we first purified capsidiol and related compounds from INF1-treated leaves of *N. benthamiana* by flash column chromatography and high-performance liquid chromatography (HPLC). The structures of purified phytoalexins capsidiol, debneyol, and capsidiol 3-acetate were identified by comparison of their nuclear magnetic resonance (NMR) data with those reported previously (Burden et al. 1986; Gordon et al. 1973; Uegaki et al. 1988) (Fig. 5A). Three major spots detected by thin-layer chromatography (TLC) corresponded to capsidiol, debneyol, and capsidiol 3-acetate (Fig. 5B). Production of capsidiol, capsidiol 3-acetate, and debneyol is reduced by silencing of *NbEAS* (Fig. 5B and C), which confirmed that NbEAS is involved in the production of phytoalexins. Production of capsidiol and capsidiol 3-acetate but not debneyol was reduced by silencing of *NbEAH* (Supplementary Fig. S3) (Fig. 5A). Given that both *NbEAS*- and *NbEAH*-silenced plants showed reduced resistance against *P. infestans* (Shibata et al. 2010), it was suggested that debneyol is not substantially involved in the resistance of *N. benthamiana* against *P. infestans*. Consistently, debneyol showed the weakest growth inhibitory activity against *P. capsici* and *P. infestans* among these three phytoalexins (M. Ojika, unpublished data).

Production of capsidiol and capsidiol 3-acetate was quantified in control or gene-silenced plants 48 h after INF1 treatment. *NbCRT2*-silenced plants produced amounts of capsidiol and capsidiol 3-acetate similar to control plants, whereas production of phytoalexins was significantly reduced in *NbCRT3a*-silenced plants (Fig. 5B and C). These results indicated that NbCRT3a but not NbCRT2 is essential for the production of phytoalexins in *N. benthamiana* induced by INF1 treatment.

UDP-glucose:glycoprotein glucosyltransferase (UGGT), NbUGGT, is not essential for the resistance against *P. infestans*.

UGGT is a folding sensor for the glycoprotein quality control cycle in ER mediated by calnexin/calreticulin. UGGT recognizes misfolded glycoproteins and returns the folding intermediates to the cycle (Ellgaard and Helenius 2003). In *Arabidopsis*, UGGT as well as CRT3 is essential for the stable accumulation of the leucine-rich repeat receptor-like kinase EFR (Li et al. 2009; Saijo et al. 2009). To elucidate whether *N. benthamiana* UGGT is required for the resistance to *P. infestans*, *NbUGGT*-silenced plants were produced. A partial sequence of *NbUGGT* was obtained from an expressed sequence tag database (accession number GO607966) and amplified by PCR to produce a silencing vector pTV00-NbUGGT. As the case of *NbCRT2* and *NbCRT3a*, two highly conserved copies of *NbUGGT* were found in the draft genome sequence of *N. benthamiana*. Suppressed expression of both *NbUGGT* genes in *NbUGGT*-silenced plants had no detectable effects on the growth of *N. benthamiana* plants (Fig. 6A).

NbUGGT-silenced plants were inoculated with *P. infestans* and disease symptoms were scored. Development of disease symptoms in *NbUGGT*-silenced plants was almost comparable with that of control plant, and this indicated that *NbUGGT* is not essential for the resistance of *N. benthamiana* to *P. infestans* (Fig. 6B). ROS production in *NbUGGT*-silenced plants was approximately 20% of that in control plants (Fig. 6C). Production of capsidiol and capsidiol 3-acetate induced by INF1 was not affected by the silencing of *NbUGGT* (Fig. 6D).

Fig. 4. Induction of cell death induced by INF1 but not by MEK2^{DD} was reduced in *NbCRT3a*-silenced *Nicotiana benthamiana*. **A**, *NbCRT2*- or *NbCRT3a*-silenced or control *N. benthamiana* plants were treated with 150 nM INF1. Photographs were taken 4 days after INF1 treatment. TRV = Tobacco rattle virus. **B**, *NbCRT2*- or *NbCRT3a*-silenced or control *N. benthamiana* plants were inoculated with *Agrobacterium tumefaciens* carrying pNPP40 (top) or pNPP40-NbMEK2^{DD}. Photographs were taken 5 days after INF1 treatment.

Altogether, NbUGGT is not essential for the resistance of *N. benthamiana* against *P. infestans*, although silencing of *NbUGGT* affected the ROS production in response to the INF1 elicitor.

Expression of *NbEAS* but not *NbPR-1a* was suppressed in *NbCRT3a*-silenced *N. benthamiana*.

The effect of gene silencing of *NbCRT3a* on the expression of various defense-related genes was investigated. Gene-silenced or control *N. benthamiana* plants were treated with INF1 and expression of *NbPR-1a* (a marker gene of SA induced genes) (Vlot et al. 2009), *NbrbohB* (a gene for NADPH oxidase for ROS production) (Yoshioka et al. 2003), *NbHsr203j* (a marker gene of HR induction) (Pontier et al. 1998), and *NbEAS* were analyzed by semi quantitative RT-PCR. Silencing of either *NbCRT2* or *NbUGGT* had no significant effect on expression of any tested defense-related genes. However, expression of the *NbEAS* gene, but not other tested genes, including SA-regulated *NbPR-1a*, was significantly reduced in *NbCRT3a*-silenced plants compared with control and *NbCRT2*- and *NbUGGT*-silenced plants (Fig. 7).

NbCRT3 is required for the ethylene production induced by INF1 elicitor.

Our previous report indicated that induced expression of genes for catalytic enzymes of phytoalexin production, *NbEAS* and *NbEAH*, were mediated by ethylene (Shibata et al. 2010). Because the expression of *NbEAS* but not *NbPR-1a* was suppressed in *NbCRT3a*-silenced plants, productivity of ethylene was compared in control and *NbCRT3a*-silenced plants. Ethylene at approximately 2 nl/g of leaf tissue/h was produced in control leaves without elicitor treatment, whereas control leaves treated with INF1 produced ethylene at 17 nl/g/h 10 h after the treatment of elicitor (Fig. 8). Leaves of *NbCRT3a*-silenced plant treated with INF1 produced only 6 nl/g/h (Fig. 8), and this indicated that NbCRT3a is involved in the ethylene production induced by INF1 treatment.

Induction of resistance reactions by crude elicitor from *P. infestans* was compromised in *NbCRT3a*-silenced *N. benthamiana*.

Production of INF1 by *P. infestans* is induced at a relatively late stage (necrotrophic phase) of the infection (Kamoun et al. 1997). Therefore, another MAMP of *P. infestans* could play a major role in the induction of resistance reactions of *N. benthamiana* in the early stage of interaction. *N. benthamiana* leaves were treated with potential elicitors in cell wall constituents of *P. infestans*, including arachidonic acid, β -glucan (laminarin), and hyphal wall component (HWC) elicitor (Doke and Tomiyama 1980). The HWC elicitor is crude elicitor extracted from mycelia of *P. infestans*, which can induce several defense reactions in potato (Takemoto et al. 1999). Among elicitors tested, only the HWC elicitor, as well as INF1, can induce the production of ROS in *N. benthamiana* leaves. The HWC elicitor also induced the production of phytoalexins whereas there is no detectable production of phytoalexins in *N. benthamiana* leaves treated with 1 mM arachidonic acid or laminarin at 1 mg/ml. Neither of the tested elicitors induced cell death of *N. benthamiana* leaves (Supplementary Fig. S4). The effect of gene silencing on HWC-induced ROS production and accumulation of phytoalexins was investigated. HWC-induced ROS production was not affected by the silencing of *NbUGGT*, whereas a significant reduction was observed in *NbCRT2*- and *NbCRT3a*-silenced plants (Fig. 9A). Production of capsidiol and capsidiol 3-acetate in *NbCRT2*- and *NbUGGT*-silenced plants was comparable with control plants, whereas *NbCRT3a*-silenced plants showed reduced production

Fig. 5. NbCRT3a is involved in the production of phytoalexins induced by INF1. **A**, Expected biosynthetic pathway of debneyol, capsidiol, and capsidiol 3-acetate in *Nicotiana benthamiana*. **B**, Phytoalexins were extracted 48 h after INF1 treatment. Samples were separated and developed on thin-layer chromatography plate. Spots for capsidiol 3-acetate, debneyol, and capsidiol are indicated by arrowheads. **C**, Phytoalexins were extracted 24 h after INF1 treatment and quantified by high-performance liquid chromatography. Data are means $\pm$ standard error ($n > 5$). Data sets 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.

of phytoalexins (Fig. 9B). These results indicated that NbCRT3a is involved in the induction of resistance reactions in response to the HWC elicitor.

DISCUSSION

Plant-specific CRT3 is required for nonhost resistance of *N. benthamiana* to *P. infestans*.

Calreticulins are lectin-like proteins, which have two major known functions, the chaperoning of glycoproteins and regulation of Ca^{2+} homeostasis in the ER lumen (Michalak et al. 1999). There are two groups of calreticulin in higher plants, CRT1/2 and CRT3 (Christensen et al. 2010; Persson et al. 2003) (Fig. 1). The growth defect of the *Arabidopsis Atcrt1b*

mutant was complemented by overexpression of *AtCRT1a* but not by *AtCRT3*, which indicated that *AtCRT1a* and *AtCRT1b* have redundant functions, whereas *AtCRT3* has a distinct role (Christensen et al. 2010). CRT1/2 is considered to have general chaperone functions in ER (Christensen et al. 2010), while a series of recent reports revealed a specialized function of CRT3 in disease resistance. *AtCRT3* was isolated by several research groups as an essential factor for the stable accumulation of the MAMP receptor EFR for the detection of elf18, derived from the bacterial elongation factor EF-Tu (Li et al. 2009; Saijo et al. 2009). Caplan and associates (2009) reported that the protein level of the induced receptor-like kinase (IRK) was reduced in *CRT3*-silenced *N. benthamiana* plants. Silencing of either *IRK* or *CRT3* compromised the *N* gene-mediated

Fig. 6. NbUGGT is not essential for the resistance of *Nicotiana benthamiana* to *Phytophthora infestans*. **A**, *N. benthamiana* plants were inoculated with Tobacco rattle virus (TRV) or TRV:NbUGGT. Expression of *NbUGGT* was assessed by reverse-transcriptase polymerase chain reaction with gene-specific primers. *NbEF-1α* was used as an internal standard. **B**, Plot showing percentage of *N. benthamiana* leaves with disease symptom severities represented in the five classes, for leaves of control or *NbCRT3a*- or *NbUGGT*-silenced plants inoculated with *P. infestans* from 3 to 5 days postinoculation. At least 10 inoculated leaves from each silenced plant were counted. **C**, Top: leaves of control or *NbUGGT*-silenced *N. benthamiana* were treated with H₂O or 150 nM INF1. Production of O_2^- was detected as L-012-mediated chemiluminescence 12 h after INF1 treatment. Circles indicate the area of treatment. Bottom: intensities of chemiluminescence were quantified with photon image processor. Data are means $\pm$ standard error ($n = 8$). **D**, Top: phytoalexins were extracted 48 h after INF1 treatment. Samples were separated and developed on thin-layer chromatography plate. Bottom: phytoalexins were extracted 24 h after INF1 treatment and quantified by high-performance liquid chromatography.

resistance against *Tobacco mosaic virus* (TMV). More recently, Liebrand and associates (2012) isolated CRT3 as an interacting protein of Cf-4, a receptor-like protein required for the resistance of tomato to a strain of *Cladosporium fulvum* producing Avr4. All these reports indicated that CRT3 is required for the stable accumulation or maturation of glycosylated cell-surface receptors for the recognition of pathogen-derived molecules.

In the present study, we identified NbCRT3a as an essential factor for the resistance of *N. benthamiana* against *P. infestans* by screening based on random gene knockdown. We were unable to directly test the role of NbCRT3a on the stability of the receptor involved in the resistance against *P. infestans*, because the receptor has not been identified. We speculated that

Fig. 7. NbCRT3a is required for the expression of *NbEAS* induced by INF1. Total RNA was isolated from *NbCRT2*-, *NbCRT3a*-, or *NbUGGT*-silenced or control *Nicotiana benthamiana* 12 h after treatment with water (H₂O) or 150 nM INF1 (INF1). Reverse-transcriptase polymerase chain reaction was performed with gene-specific primers. Constitutively expressing *NbEF-1α* was used as an internal standard. Results shown are representative of at least three separate experiments. TRV = *Tobacco rattle virus*.

Fig. 8. Ethylene production induced by INF1 was suppressed in a *NbCRT3a*-silenced plant. Leaves of control or *NbCRT3a*-silenced *Nicotiana benthamiana* were treated with water (H₂O) or 150 nM INF1 and ethylene production from treated leaves was quantified by gas chromatography. Results shown are representative of three separate experiments. TRV = *Tobacco rattle virus*.

NbIRK for N-mediated TMV resistance (Caplan et al. 2009) and NbLRK1 (lectin-like protein kinase 1), identified as INF1 interaction protein (Kanzaki et al. 2008), were potential targets of NbCRT3a for resistance against *P. infestans*. We made *NbIRK*-silenced or *NbLRK1*-silenced plants; however, our preliminary tests indicated that those receptor-like kinases are not involved in the resistance of *N. benthamiana* to *P. infestans* (M. Matsukawa and D. Takemoto, unpublished data). Given

Fig. 9. NbCRT3a is involved in the phytoalexin accumulation and reactive oxygen species production of *Nicotiana benthamiana* induced by hyphal cell wall (HWC) elicitor. **A**, Phytoalexins were extracted from leaves of control or *NbCRT2*-, *NbCRT3a*-, or *NbUGGT*-silenced *N. benthamiana* 72 h after HWC treatment, and quantified by high-performance liquid chromatography. Data are means $\pm$ standard error (SE) ($n > 5$). Data sets 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. **B**, Top: leaves of control or gene-silenced *N. benthamiana* were treated with H₂O or 150 nM INF1. Production of O₂⁻ was detected as L-012-mediated chemiluminescence 12 h after INF1 treatment. Circles indicate the area of treatment. Bottom: intensities of chemiluminescence were quantified with photon image processor. Data are means $\pm$ SE ($n = 8$).

that cell death induction by MEK2^{DD} was not affected in *NbCRT3a*-silenced plants, it was expected that *NbCRT3a* would be involved in an early step of signal transduction for disease resistance, probably the maturation of unidentified receptors of INF1 or MAMPs derived from *P. infestans* (Fig. 10).

***NbCRT3a* is involved in the activation of signal transduction for the induction of ethylene production.**

We have previously reported that silencing of either isochorismate synthase 1 for SA biosynthesis (*NbICS1*) (Wildermuth et al. 2001) or ethylene insensitive 2 for ethylene signaling (*NbEIN2*) (Alonso et al. 1999) compromised the resistance of *N. benthamiana* to *P. infestans*. Expression of *NbEAS* was suppressed in *NbEIN2*-silenced plants but not by silencing of *NbICS1*, which indicated that production of capsidiol is predominantly controlled via ethylene-mediated signaling in *N. benthamiana* (Shibata et al. 2010). Silencing of *NbEIN2* also compromised ROS production in response to INF1 treatment (Shibata et al. 2010). *NbCRT3a*-silenced plants showed reduced accumulation of phytoalexins and ROS production (Figs. 3 and 5), which is consistent with the reduced production of ethylene in *NbCRT3a*-silenced plants (Figs. 8 and 10). In contrast, expression of *NbPR-1a*, a marker gene for SA-mediated signaling, was not affected by the silencing of *NbCRT3a* (Fig. 7). Altogether, compromised resistance of *NbCRT3a*-silenced plants is a consequence of the reduced production of ethylene. In our VIGS-based screening for genes required for the resistance against *P. infestans*, we identified several genes of catalytic enzymes for ethylene production or phytoalexin production (i.e., genes for enzymes in the mevalonate pathway) but not a single known gene related to SA or jasmonic acid signaling. Therefore, ethylene-mediated production of phytoalexins probably plays a central role in the resistance of *N. benthamiana* against *P. infestans*.

***NbCRT2*-silenced *N. benthamiana* showed reduced defense reactions but *NbCRT2* was not essential for the resistance against *P. infestans*.**

Although previous reports consistently indicated that CRT3 played a major role in the chaperoning of the glycosylated receptor proteins for disease resistance, partial involvement of another member of CRT in plant disease resistance has been reported. Caplan and associates (2009) reported that silencing of *CRT3* caused reduced accumulation of IRK, while silencing of *CRT2* also had a relatively minor but significant effect on the accumulation of IRK as well as resistance against TMV mediated by IRK. In our experiment, *NbCRT2* was initially isolated from the plant line A3-199, which showed weak disease symptoms caused by *P. infestans*. Although we could not reproduce the result showing reduced resistance in *NbCRT2*-silenced plants, *CRT2* may be partially involved in the resistance against *P. infestans* in particular environmental conditions.

***NbUGGT* is not essential for the resistance of *N. benthamiana* against *P. infestans*.**

In the present study, we have shown that UGGT of *N. benthamiana* is dispensable for the resistance against *P. infestans* (Fig. 6B). This result is contradictory to the previous reports with *Arabidopsis*, which showed that UGGT was required for the stable accumulation and function of EFR (Li et al. 2009; Saijo et al. 2009). Although silencing of *NbUGGT* had no detectable effects on resistance against *P. infestans*, production of ROS in response to INF1 treatment was affected as the case of *NbCRT2* silencing (Figs. 3 and 6). Generally, CRT are known as the chaperone required for the correct folding of the glycoproteins, whereas the primary role of UGGT is the recycling of incompletely folded glycoproteins (Ellgaard and Helenius

2003). Therefore, the observed dispensability of UGGT could be explained by the idea that glycoproteins being correctly folded by CRT3a and co-chaperones are sufficient to induce resistance against *P. infestans* without more efficient ERQC with UGGT, at least in our experimental conditions.

***NbCRT3a* is required for the reactions to MAMP elicitor derived from *P. infestans*.**

Mature *N. benthamiana* plants show strong resistance to *P. infestans*, whereas young *N. benthamiana* are susceptible to this pathogen (Shibata et al. 2011). Microscopic observation of resistant and susceptible interactions revealed that differences of these interactions were obvious within 24 h after inoculation (Shibata et al. 2010). Given that the production of INF1 by *P. infestans* is downregulated in early stages of infection (Kamoun et al. 1997), INF1 would not play a critical role in early stages of the interaction between host plants and *P. infestans*. Another potential elicitor involved in the interaction between *N. benthamiana* and *P. infestans* would be cell wall or membrane constituents of *P. infestans*, such as polysaccharides and fatty acids. In the present study, β -1,3-glucan (laminarin) and arachidonic acid were used as candidate MAMPs of *P. infestans*. However, neither of them induced detectable resistance reactions in *N. benthamiana*, even though we used them at relatively higher concentrations compared with that in previous reports. Treatment of the crude HWC elicitor prepared from *P. infestans* induced ROS production and accumulation of phytoalexins. Although elicitor molecules in the HWC have not been identified, the procedure of HWC preparation (see Materials and Methods) indicates that the HWC includes polysaccharides, probably cell wall constituents of oomycetes such as glucans and cellulose (Bartnicki-Garcia 1968), and doesn't

Fig. 10. Model for the role of CRT3 in resistance of *Nicotiana benthamiana* against *Phytophthora infestans*. CRT3 is required for the induction of elicitor-induced but not MEK2-induced production of ethylene (ET). EIN2 is required for the resistance of mature *N. benthamiana* against *P. infestans* as well as production of reactive oxygen species (ROS). Expression of *EAS* and *EAH*, genes for capsidiol biosynthesis, is upregulated by ET and required for resistance of *N. benthamiana* to *P. infestans*. FPP = farnesyl pyrophosphate.

contain proteins such as INF1. The silencing of *NbCRT3a* suppressed HWC-induced resistance reactions; therefore, *NbCRT3a* could be involved in the maturation of receptors of unidentified elicitor molecules in HWC.

In summary, the present study indicated that plant-specific calreticulin *NbCRT3a* of *N. benthamiana* is essential for the resistance against *P. infestans*. *NbCRT3a* is involved in the induction of ethylene production in response to the pathogen attack, which is required for the production of phytoalexins. Based on previous reports (Caplan et al. 2009; Li et al. 2009; Liebrand et al. 2012; Saijo et al. 2009), the expected function of *NbCRT3a* in *N. benthamiana* is the chaperoning of the receptor or receptors for the recognition of elicitors derived from *P. infestans*. However, CRT are known as multifunctional proteins (Michalak et al. 1999). The most recent report showed that root-knot nematodes secrete Mi-CRT, a calreticulin derived from nematodes, which plays an important role in infection success (Jaouannet et al. 2013), indicating that there could be unexpected functions of CRT in plant-microbe interactions. Therefore, further investigations, including isolation of receptors of INF1 and elicitor molecules in HWC, is required to reveal the role of *NbCRT3a* in the resistance of *N. benthamiana* against *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* isolate 08YD1 (Shibata et al. 2011) was maintained on rye media at 20°C. Zoospore suspensions of *P. infestans* were prepared as follows. *P. infestans* isolates were subcultured on rye-media for 7 to 10 days. Water (20 ml) was added to the surface of the *P. infestans* colonies, which were then rubbed with a cotton swab to release the zoospores. Induction of zoospores was performed as described previously (Doke 1975). Leaves of mature *N. benthamiana* (approximately 35 days from the date of sowing) 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 postinoculation, then moved to a growth room at 23°C.

Preparation of elicitors.

INF1 elicitor was prepared as follows. *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 supplemented 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-kD molecular mass cutoff; Pierce Biotechnology, Rockford, IL, U.S.A.) overnight at 4°C. Note that mutated INF1, INF1 S3, prepared from *E. coli* carrying pFB52 (Kamoun et al. 1997) by the same procedure, did not induce resistance reactions (Supplementary Fig. S5), and confirmed that resistance reactions observed in this study are induced by elicitor activity of INF1.

HWC elicitor was prepared from cultured mycelia of *P. infestans* isolate PI1234-1 according to the method previously described (Doke and Tomiyama 1980). Mycelia (30 g, 2 weeks old) of *P. infestans* grown in rye media were ground in liquid nitrogen with a mortar and pestle and mixed with 150 ml of 0.05 M acetate buffer (pH 4.5). The mixture was homogenized

in a Waring blender, sonicated at 4°C with a sonicator (Model W-225R; Heat Systems Ultrasonics Inc., Plainview, NY, U.S.A.), and centrifuged at 14,000 $\times$ g for 30 min. The pellet was suspended in 2 volumes of 0.1 M borate buffer (pH 8.8), homogenized in a Waring blender, and sonicated at 4°C. The homogenate was autoclaved at 120°C for 15 min and centrifuged at 14,000 $\times$ g for 30 min. The supernatant was dialyzed against distilled water with a dialysis tube (molecular weight cut-off = 12,000) and mixed with an equal volume of ether. A water-soluble fraction was collected and lyophilized. The lyophilized powder was used as the HWC elicitor.

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, Japan) and PCR was performed with GoTaq Master Mix (Promega Corp., Madison, WI, U.S.A.). Gene-specific primer combinations (Supplementary Table S1) used for expression analysis were as follows: *NbCRT2*-F and *NbCRT2*-R (*NbCRT2*), *NbCRT3*-F2 and *NbCRT3*-R1 (*NbCRT3*), *BI*-*NbUGGT*-F and *NbUGGT*-R2 (*NbUGGT*), *GE*-*PR-1a*-F and *PR-1a*-R (*NbPR-1a*), *GE*-*HSR203J*-F and *GE*-*HSR203J*-R (*Nbhsr203j*), *RBOHB*-F and *RBOHB*-R (*NbRbohB*), *EAS*-F-H and *GE*-*EAS*-R (*NbEAS*), and *EF1a*-F and *EF1a*-R (*NbEF1 α*). The PCR products were separated on a 1.8% agarose gel and visualized under UV illumination after ethidium bromide staining.

Preparation of VIGS library.

cDNA fragments cloned in pTV00 were prepared using PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, U.S.A.) according to the protocol from the manufacturer with minor modifications. Leaves of *N. benthamiana* were treated with 150 nM INF1; harvested 1, 3, 6 and 12 h after the treatment; and subjected to the preparation of "tester" cDNA. Leaves of *N. benthamiana* without treatment was used for the preparation of "driver" cDNA. Instead of using nested primers provided with the kit, BI library primer and HIII library primer were used for the second PCR. PCR product was digested with *Bam*HI and *Hind*III and cloned into the *Bam*HI/*Hind*III site of pTV00. Vectors with random cDNA fragments were transformed into *A. tumefaciens* (strain GV3101 with helper plasmid pSoup) by electroporation with a MicroPulser electroporator (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and transformants were selected on LB media supplemented with rifampicin at 50 μ g/ml, kanamycin at 50 μ g/ml, and tetracycline at 2.5 μ g/ml.

Construction of vectors.

Expression vector pNPP40 was prepared by cloning a 0.8-kb *Sac*I/*Xho*I-digested fragment of CaMV 35S promoter and Ω sequence amplified from pEl2 Ω vector (Ohtsubo et al. 1999) with primers *Sac*I-El2-F and pEl2-R into the *Sac*I/*Xho*I site of pTV00. pNPP40-NbMEK2^{DD} vector was prepared as follows. A 0.6-kb 5' fragment NbMEK2 was amplified with primers SpI-MEK2^{DD}-F and MEK2^{DD}-Xh-R from cDNA of *N. benthamiana* and cloned in the *Eco*RV site of pBluescript by TA cloning. A 0.5-kb 3' fragment NbMEK2^{DD} was amplified with MEK2^{DD}-F3 and MEK2^{DD}-SpI-R, followed by PCR with MEK2^{DD}-F2 and MEK2^{DD}-SpI-R, and MEK2^{DD}-F1 and MEK2^{DD}-SpI-R to introduce substitution for constitutive active form. Amplified PCR product was cloned in the *Eco*RV site of pBluescript by TA cloning. The 5' and 3' fragments of NbMEK2 were then excised from the vectors by *Xba*I/*Xho*I and *Xho*I/*Eco*RI, respectively, and cloned into the *Xba*I/*Eco*RI site of pNPP40.

To produce silencing vector of NbUGGT, partial cDNA of NbUGGT were amplified from *N. benthamiana* cDNA using gene-specific primers BI-NbUGGT-F and NbUGGT-H-R. An amplified fragment was digested with *Bam*HI and *Hind*III and cloned into the *Bam*HI/*Hind*III site of pTV00.

For all constructs, the inserts were sequenced to confirm cloning of the correct gene fragment. The pNPP40 or pTV00 vectors were transformed into *A. tumefaciens* (strain GV3101 with helper plasmid pSoup) as described above.

Expression vector pNPP30 was prepared by cloning an *Hind*III/*Nde*I-digested fragment of CaMV 35S promoter and Ω sequence amplified from pEI2 Ω vector (Ohtsubo et al. 1999) with primers pEI2-F2 and Tnos-Nde-R into the *Hind*III/*Nde*I site of pUC19 (Yanisch-Perron et al. 1985). Vector pNPP30-GFP was prepared by cloning a *Bam*HI/*Spe*I-digested fragment of enhanced green fluorescent protein (EGFP) amplified from pNPP5 (Takemoto et al. 2011) with primers B-EGFP-F and EGFP-Sp-R into the *Bam*HI/*Spe*I site of pNPP30. Vector pNPP30-GFP-3GA was prepared by cloning a *Bam*HI/*Eco*RI-digested GFP-3GA fragment amplified from pNPP30-GFP with primers B-EGFP-F and GFP-3GA-EI-R into the *Bam*I/*Eco*RI site of pNPP30. The GFP-3GA has three copies of glycine-alanine (GA) at the C terminus of the GFP to act as a spacer between EGFP and the test protein. For subcellular localization analysis, full-length NbCRT2a and NbCRT3a2 were amplified with primers IF-3GA-CRT2a-F and IF-3GA-CRT2a-R or IF-3GA-CRT3a2-F and IF-3GA-CRT3a2-R from cDNA of *N. benthamiana* and cloned into the *Eco*RI/*Spe*I site of pNPP30-3GA-GFP with the In-Fusion HD Cloning kit (Clontech) to generate pNPP30-NbCRT2a-GFP and pNPP30-NbCRT3a2-GFP.

Transient gene expression

by agroinfiltration and particle bombardment.

A. tumefaciens carrying the pNPP40 or pNPP40-NbMEK2^{DD} construct were 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 in the intercellular space of *N. benthamiana* leaves using a syringe without a needle.

Particle bombardments were carried out using a Bio-Rad PDS-1000/He biolistic particle delivery system (Bio-Rad Laboratories) as previously described (Takemoto et al. 2012). pNPP30-GFP, pNPP30-GFP-NbCRT2, or pNPP30-GFP-NbCRT3 vector (1 μ g) was used to coat 1.25 μ g of 1- μ m-diameter gold particles in a 25- μ l suspension. Transformed epidermal cells of *N. benthamiana* were monitored by confocal laser-scanning microscopy 9 h after bombardments. Confocal fluorescence images were captured using a confocal laser-scanning microscope (FV1000-D; Olympus, Tokyo). The laser was used as a 488-nm excitation source, and GFP fluorescence was recorded between 495 and 520 nm.

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 media. Bacterial suspensions were then collected by centrifugation at $16,000 \times g$ for 1 min. The bacterial cells were then resuspended in 10 mM 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. After 3 to 4 weeks of infiltration, upper leaves of the inoculated plants were used for experiments.

ROS measurement.

The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence as reported previously (Uruma et al. 2009). L-012 (Wako, Kyoto, Japan) is a luminol derivative that has a highly sensitive to superoxide radicals. 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 into the intercellular space of leaves using a needleless syringe. 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, Hamamatsu City, Japan), and quantified using the U7501 program (Hamamatsu Photonics).

Isolation of phytoalexins.

Leaves of *N. benthamiana* (65 g, fresh weight) treated with elicitor were homogenized in 200 ml of 50% methanol. The mixture was filtered through a filter paper, and the filtrate was extracted three times with 100 ml of hexane/ethyl acetate (1:1, vol/vol). The residue on the filter paper was washed with 100 ml of 67% methanol, and the filtrate was extracted three times with 100 ml of hexane/ethyl acetate (1:1, vol/vol). All the hexane/ethyl acetate extracts were combined and concentrated in vacuo to give an oily material (87 mg). The oily material was separated by flash column chromatography (Hi-Flash column, S-size, 6 g of silica gel; Yamazen Science, Osaka, Japan), with linear gradient of 10 to 80% ethyl acetate in hexane to give 11 fractions. The fifth fraction (5.0 mg), which eluted at 30 to 34% ethyl acetate in hexane, was purified by HPLC (Develosil ODS-UG-5, 10 by 250 mm, 55% acetonitrile, 3 ml/min, detection at 210 nm) to give capsidiol 3-acetate (1.9 mg, t_R = 37.0 min). The eighth fraction (13.2 mg), which eluted with 44 to 56% ethyl acetate in hexane, was purified by HPLC (Develosil ODS-UG-5, 10 by 250 mm, 50% acetonitrile, 4 ml/min, detection at 217 nm) to give debneyol (9.3 mg, t_R = 21.2 min). The tenth fraction (7.7 mg), which eluted with 80% ethyl acetate in hexane, was purified by HPLC (Develosil ODS-UG-5, 10 by 250 mm, 65% methanol, 3 ml/min, detection at 210 nm) to give capsidiol (1.3 mg, t_R = 26.5 min). The structures of phytoalexins were identified by comparison of their NMR data with those reported previously (Burden et al. 1986; Gordon et al. 1973; Uegaki et al. 1988).

Detection of phytoalexins by TLC.

Leaves of *N. benthamiana* (300 mg) were ground in liquid nitrogen with a mortar and pestle, and suspended in 1 ml of 50% methanol. The mixture was centrifuged at $16,000 \times g$ for 1 min, and the supernatant was mixed with cyclohexane/ethyl acetate (1:1, vol/vol). The mixture was centrifuged at $3,000 \times g$ for 5 min, and the upper phase (ethyl acetate layer) was collected and dried up using a rotary evaporator. Resultant precipitate was suspended in 15 μ l of methanol and subjected to TLC. Extracted samples (15 μ l/lane) were developed on TLC plates (TLC aluminum sheet of silica gel 60; Merck, NJ, U.S.A.) with cyclohexane/ethyl acetate (1:1, vol/vol) and visualized by spraying with 2.8% vanillin and 0.5% sulfuric acid in methanol, followed by heating at 120°C.

Quantitative analysis of phytoalexins by HPLC.

Crude phytoalexins are extracted from leaves of *N. benthamiana* (300 mg) treated with elicitors as described above. The dried extract in a 1.5-ml microtube was dissolved in 150 μ l

of 67% acetonitrile, and a 5- μ l portion (corresponding to 10 mg of tobacco leaves) was subjected to HPLC analysis under the following conditions. Column: Develosil ODS-UG-3 (4.6 by 150 mm); solvent: 50% acetonitrile (0 to 3 min), 50 to 80% acetonitrile (3 to 13 min), and 80 to 100% acetonitrile (13 to 14 min); flow rate: 1.0 ml/min; detection at 205 nm. Capsidiol and capsidiol 3-acetate eluted at 4.6 and 12.8 min, respectively. The peak areas were used for quantitative analysis.

Quantitative analysis of ethylene production by gas chromatography.

Five pieces of *N. benthamiana* leaf tissue were excised with a cork borer (10 mm in diameter) and placed in a 5-ml gas chromatography (GC) vial sealed with a rubber plunger. After 3 h of incubation, a 1-ml air sample in the vial was collected using a grass syringe. Quantity of ethylene in collected air was determined using a gas chromatograph (GC-353) equipped with a flame thermionic detector (GL Sciences, Tokyo) and CP-PoraBOND Q column (Varian Inc., Middelburg, The Netherlands). GC was performed with column temperature at 50°C, injection temperature at 120°C, and detector temperature at 150°C using N₂ as the carrier gas.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Sol Genomics Network database:
solgenomics.net/organism/Nicotiana_benthamiana/genome