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SGT1 and *HSP90* are essential for age-related non-host resistance of *Nicotiana benthamiana* against the oomycete pathogen *Phytophthora infestans*

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ABSTRACT

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. We investigated the interaction between *P. infestans* and Solanaceous model plant *Nicotiana benthamiana*. Mature *N. benthamiana* plants were resistant to 8 isolates of *P. infestans*, whereas relatively young plants were susceptible to all isolates. Analysis with virus-induced gene silencing (VIGS) indicated that *NbSGT1* and *NbHSP90*, genes essential for the function of R proteins, are required for the resistance of *N. benthamiana* to *P. infestans*. *NbSGT1* was also required for the production of reactive oxygen species (ROS), hypersensitive cell death and expression of *NbEAS*, a gene for phytoalexin biosynthesis, induced by INF1, a secretory protein derived from *P. infestans*. These results suggested that *N. benthamiana* possibly possesses a broad-spectrum R protein against *P. infestans*, which requires an SGT1/HSP90-dependent mechanism, for the recognition of a conserved molecular pattern of *P. infestans*.

1. Introduction

Phytophthora infestans is an oomycete pathogen that causes potato late blight, one of the most destructive and economically important plant diseases. This pathogen is historically significant as a causal agent of the Irish potato famine in the 1840s. Even today, the control efforts and yield losses related to this pathogen amount to more than $3 billion per year worldwide [1]. About 10 dominant race-specific disease resistance (*R*) genes have been identified in the wild species *Solanum demissum*, several of which have been introduced into cultivated potato varieties. However, resistance dependent on these *R* genes were quickly defeated by emerging new races of *P. infestans* [1,2]. More recently, *R* genes for broad-spectrum resistance to *P. infestans* have been identified from several wild *Solanum* species, including *RB/Rpi-blb1* and *Rpi-blb2* from *Solanum bulbocastanum* [3–5], *Rpi-pnt1* from *Solanum pinnatisectum* [6] and *Rpi-moc1* from *Solanum mochiquense* [7]. Introduction of these *Rpi* (Resistance to *P. infestans*) genes into potato varieties is considered the most practical approach to achieve durable resistance of potato plants against *P. infestans*.

Recognition of molecules derived from potential pathogens is a crucial step for plants to induce effective disease resistance. Structural molecules from plant pathogens, such as fungal cell wall constituents (chitin, glucan, protein and glycoprotein) and bacterial lipopolysaccharide (LPS) and flagellin, elicit defense responses in a wide range of plant species. Such nonspecific elicitors are termed microorganism-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), which are recognized for the induction of innate immunity of plants [8]. Another group of elicitors are recognized by a specific range of plant species or cultivars. *P. infestans* produces a large number of secretory proteins during the process of infection, including apoplastic (e.g. INF1 and NPP1.1, [9,10]) and intracellular elicitors (e.g. AVR3a and ipiO, [11,12]). Elicitins, major secretory proteins produced by *Phytophthora* and *Pythium* species, are sterol-carrier proteins that enable the pathogen to uptake sterol from plant plasma membranes and also act as PAMPs of oomycete pathogens recognized by a limited number of dicot plants including *Nicotiana* species [13–15]. *P. infestans* has at least 7 elicitin-like genes, including INF1 that is abundantly produced and commonly used as an elicitor for induction of disease resistance in *Nicotiana* species [16,17]. *P. infestans* AVR3a, which induces resistance reactions in potato carrying corresponding R protein R3a [11], has an RXLR motif for translocation of secreted protein into plant cells. AVR3a also contributes to virulence

Abbreviations: EDS1, Enhanced disease susceptibility 1; HSP90, Heat shock protein 90; NDR1, Non-race-specific disease resistance 1; RAR1, required for Mla12 resistance 1; ROS, reactive oxygen species; SGT1, Suppressor of the G2 allele of *skp1*; VIGS, Virus-induced gene silencing.

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of this pathogen by suppressing the PAMP-induced resistance reactions [18]. Recent reports revealed that the avirulence factors for RB/Rpi-blb1 and Rpi-blb2 are RXLR effectors (Avrblb1 (ipiO) and Avrblb2), which are highly conserved within *P. infestans* isolates [12,19]. These reports indicate that broad-spectrum resistance to *P. infestans* by Rpi proteins is induced by mechanisms similar to R protein-mediated induction of disease resistance.

Recently, several factors commonly required for R protein-mediated resistance have been identified. RAR1 (required for Mla12 resistance 1) was originally isolated form barley as a gene required for powdery mildew resistance determined by many genes at the *Mla* locus [20]. Arabidopsis *rar1* mutants have been independently isolated by several groups using genetic screens for loss of resistance mediated by different *R* genes, *RPS5*, *RPP5* or *RPM1* [21–23]. SGT1 (Suppressor of the G2 allele of *skp1*) was identified as an interactor of RAR1 by yeast two-hybrid screens, and it was shown that RAR1 and SGT1 form a complex *in vivo* [24,25]. Double-stranded RNA mediated gene silencing of either *SGT1* or *RAR1* in barley cells showed loss of *Mla6*-dependent resistance, suggesting that SGT1 and RAR1 together play key roles in the function of Mla6 [24]. In contrast to one *SGT1* gene in barley, there are two *SGT1* genes in Arabidopsis, and only *SGT1b*, but not *SGT1a*, is essential for the induction of disease resistance via several R proteins, such as RPP2, RPP4 and RPP6 [26,27]. HSP90 (Heat shock protein 90) is an interactor of both SGT1 and RAR1 [28,29]. Arabidopsis has four *HSP90* genes; *HSP90.1* has been shown to be required for *RPS2*-dependent resistance to bacterial pathogen as well as *RLM1*- and *RLM2*-dependent resistance to blackleg disease [28,30]. There are a limited number of reports about loss of function of Arabidopsis R proteins by knockout of a *HSP90* gene, probably because of the redundancy of the highly conserved four Arabidopsis *HSP90* genes. Gene silencing of the *HSP90* gene, which could reduce the expression level of multiple copies of conserved *HSP90* genes, compromised *Mla*-dependent barley resistance to powdery mildew, *Mi*- and *I2*-dependent tomato resistance to nematode and *Fusarium oxysporum*, respectively, and *R3a*-dependent potato resistance to *P. infestans* [18,31–33]. HSP90 is a conserved molecular chaperone in eukaryotic cells and is known to be involved in the stabilization, maturation and assembly of proteins for intracellular signal transduction. A series of reports indicate that silencing or knockdown of *RAR1*, *SGT1* or *HSP90* causes the reduction of R proteins, suggesting that one of the functions of HSP90-SGT1-RAR1 chaperone complex is stabilization of R proteins [34–36].

Genetic screens for loss of function of R proteins identified *EDS1* (Enhanced disease susceptibility 1) and *NDR1* (Non-race-specific disease resistance 1), which encode lipase-like protein and probable membrane binding protein, respectively [37]. Interestingly, EDS1 and NDR1 are generally required for the function of TIR (Toll/Interleukin-1 receptor)-NB-LRR and CC (coiled coil)-NB-LRR class of R proteins, respectively, although a few NB-LRR class R proteins, including RPP8 and RPP13, require neither of the two [37–39]. Exact functions of EDS1 and NDR1 in signal transduction have not been revealed yet.

Although *P. infestans* is a practically important pathogen, genes required for plant resistance against *P. infestans* are largely unknown except *R* genes, perhaps because of the time consuming processes for the construction of genetically modified potato. Most Solanaceous species, except potato and tomato, are generally resistant to *P. infestans*. *Nicotiana benthamiana*, a Solanaceous model plant, is a good candidate as a model host plant of *P. infestans*, as there are well-established methods for functional analysis of genes by *Agrobacterium*-mediated transient gene expression or virus-induced gene silencing (VIGS) [40]. However, *N. benthamiana* has been reported both as a resistant [41,42] as well as a susceptible [43,44] plant to *P. infestans*.

The objectives of this research were: (1) to elucidate the interaction between various isolates of *P. infestans* and *N. benthamiana*, and (2) to identify the essential genes for *N. benthamiana* resistance to *P. infestans* infection. Here we focus on the role of *N. benthamiana* genes homologous known to be required for R protein-mediated induction of disease resistance in other species, such as *SGT1*, *RAR1*, *HSP90*, *NDR1* and *EDS1*, of *N. benthamiana* against *P. infestans*.

2. Materials and methods

2.1. 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 (*Solanum tuberosum*) tubers (isolates PI0-1 and PI1234-1), or on rye-media (isolates NE0805, IG0803, MR0803, 08YB1, 08YD1 and 08MB), at 20 °C. Races of isolates PI0-1 (race 0) and PI1234-1 (race 1.2.3.4) were determined by pathogenicity tests with potato cultivars with/without *R1*, *R2*, *R3* and/or *R4*. Race of isolates 08YB1, 08YD1 and 08MB (race 1.3.4.10) were determined by inoculating potato cultivars with/without *R1*, *R2*, *R3*, *R4*, *R6* and/or *R10* with isolates. Races of isolates NE0805, IG0803 and MR0803 have not been determined. Collection of zoosporangia and induction of zoospore production from *P. infestans* PI0-1 or PI1234-1 was performed as described by Doke et al. [45]. Zoosporangia suspensions from the remaining *P. infestans* isolates were prepared as follows. *P. infestans* isolates were sub-cultured on rye-media for 7–10 days. 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 [45]. Leaves of *N. benthamiana* plants were inoculated with 1 ml aliquots 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 post inoculation, and then moved to a growth room at 23 °C.

2.2. Preparation and treatment of INF1 elicitor

Escherichia coli cells (DH5α) carrying a chimeric plasmid containing inf1, pFB53 [9], were cultured overnight at 37 °C, diluted (1:100) in Luria-Bertani (LB) medium supplemented with 50 μg/ml ampicillin, and grown until the OD_{600} of the culture reached 0.6. Production of INF1 was induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3–4 h. The culture was centrifuged and the resultant supernatant was dialyzed against water with SnakeSkin dialysis tubing (7 kD molecular mass cutoff, Pierce Biotechnology) overnight at 4 °C.

2.3. DNA sequencing and bioinformatics

DNA sequencing utilized the dideoxynucleotide chain termination method using Big-Dye (version 3) chemistry (Applied BioSystems) and the separation of products on an ABI3130 analyser (Applied BioSystems). Sequence data was analyzed and annotated in MacVector ver. 10.6 (MacVector Inc., Cary, NC, USA) or GENETYX ver. 7 (GENETYX Corp., Tokyo, Japan). Sequences of cDNA for *NbSGT1* (Accession No. AF516180 [46]), *NbHSP90-1* (AY368904 [29]), *NbEDS1* (AF479625 [47]), *NbNDR1* (AY438029 [48]), *NbrbohB* (AB079499 [42]) have been published. Sequence of cDNA for *NbRAR1* was obtained from the *N. benthamiana* EST database (TGI database, Sequence ID: TC14452) by searching with the sequence of *Nicotiana tabacum RAR1* (AF480487 [25]). Sequence of cDNA for *NbEAS* was obtained from the *N. benthamiana* EST database (PlantGDB, Sequence ID: PUT-173a-Nicotiana_benthamiana-1 17394) by searching with the sequence of *N. tabacum EAS* (AY313939 [49]). Partial cDNA for *NbPR1a* and *Nbhsr203j* were amplified from

N. benthamiana cDNA with specific primers designed from *N. tabacum* sequences for *PR1a* (X12485 [50]) and *hsr203j* (X77136 [51]).

2.4. RT-PCR

Total RNAs were isolated from *N. benthamiana* leaves using TRIzol Reagent (Invitrogen), according to the manufacturer's recommendations. cDNA synthesis was conducted using a commercial kit (ReverTra Ace -α-, TOYOBO) and PCR was performed with GoTaq Master Mix (Promega). The thermocycle conditions used were: one cycle of 94 °C for 2 min; 'x' cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min (per kb); one cycle of 72 °C for 5 min. Gene-specific primer combinations (Supplementary Table 1) used and the number of PCR cycles for expression analysis were as follows: 25 cycles for primer pairs NbSGT1-GE-F and NbSGT1-R (*NbSGT1*), NbRAR1-F and NbRAR1-GE-R (*NbRAR1*), NbHSP90-F and NbHSP90-1-GE-R (*NbHSP90*), GE-PR-1a-F and PR-1a-R (*NbPR-1a*), and GE-HSR203J-F and GE-HSR203J-R (*Nbhsr203j*), 18 cycles for primer pair EAS-F-H and GE-EAS-R (*NbEAS*) and 23 cycles for primer pair EF1a-F and EF1a-R (*NbEF-1α*). The PCR products were separated on a 1.8% agarose gel and were visualized under UV illumination after ethidium bromide staining.

2.5. Preparation of silencing constructs

Partial cDNAs of *NbSGT1*, *NbRAR1*, *NbHSP90*, *NbEDS*1 and *NbNDR*1 were amplified from *N. benthamiana* cDNA using gene-specific primers. The gene-specific primer combinations (Supplementary Table 1) used to construct the silencing vectors were as follows: NbSGT1-F and NbSGT1-R (*NbSGT1*), NbRAR1-F and NbRAR1-R (*NbRAR1*), NbHSP90-F and NbHSP90-R (*NbHSP90*), NbEDS1-F and NbEDS1-R (*NbEDS1*), and NbNDR1-F and NbNDR1-R (*NbNDR1*). Amplified fragments were ligated into *Sma*I site of the pTV00 vector [40] to generate pTV00:SGT1, pTV00:RAR1, pTV00:HSP90, pTV00:EDS1 and pTV00:NDR1. For all constructs, the inserts were amplified and sequenced to confirm cloning of the correct gene fragment. The pTV00 vectors were transformed into *Agrobacterium tumefaciens* (strain GV3101 with helper plasmid pSoup) by electroporation with a MicroPulser electroporator (Bio Rad) and transformants were selected on LB media supplemented with 50 μg/ml rifampicin, 50 μg/ml kanamycin and 2.5 μg/ml tetracycline.

2.6. Virus-induced gene silencing (VIGS)

Induction of VIGS was performed as previously reported [40]. *A. tumefaciens* GV3101 carrying the binary TRV RNA 1 construct pBINTRA6, and the TRV RNA2 vector pTV00 or pTV00 derivatives, were cultured to saturation in LB media. Bacterial suspensions were then collected by centrifugation at 16,000 × g for 1 min. The bacterial cells were then resuspended in 10 mM MES-NaOH (pH 5.6), 10 mM $MgCl_2$ and 150 μM acetosyringone (final $OD_{600} = 0.5$), and incubated at room temperature for 2 h. The cultures were mixed in a 1:1 ratio (RNA1/RNA2), and used to infiltrate into the intercellular space of leaves of *N. benthamiana* from the backside of the leaf using a syringe without a needle with placing a finger from the other side of the leaf. After three to four weeks of infiltration, the upper leaves of the inoculated plants were used for experiments. Plants inoculated with *A. tumefaciens* carrying pBINTRA6 and pTV00 were used as negative controls of the experiments.

2.7. ROS measurement

The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence as reported previously [52]. L-012 (Wako, Japan) is a luminol derivative that is highly sensitive to superoxide radicals. To detect the ROS production in *N. benthamiana* leaves, 0.5 mM L-012 in 10 mM MOPS (3-Morpholinopropanesulfonic acid)-KOH (pH 7.4) was infiltrated into the intercellular space of leaves using 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, Japan), and quantified using the U7501 program (Hamamatsu Photonics, Japan).

2.8. 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 et al. [53] 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 H_2O, 10 ml lactic acid, 10 ml glycerol, 10 g phenol, and 10 mg trypan blue). After the leaves had been allowed to cool at room temperature for 1 h, the stain was replaced with 1 g/m1 chloral hydrate. Stained leaves were monitored using an Olympus microscope BX51 (Olympus).

3. Results and discussion

3.1. Mature N. benthamiana is resistant to P. infestans isolates

To elucidate the interaction between *N. benthamiana* and *P. infestans*, *N. benthamiana* plants, at different stages of growth, were inoculated with *P. infestans* isolates PI0-1 (race 0), PI1234-1 (race 1.2.3.4), 08YD1 (race 1.3.4.10), 08YB1 (race 1.3.4.10), 08MB (race 1.3.4.10), IG0803, MR0803S or NE0805. Within 10 days post inoculation with *P. infestans* isolates, relatively young plants (20 days from the date of sowing) showed water-soaked disease symptoms on inoculated leaves (Fig. 1). In contrast, mature *N. benthamiana* plants (35 days old) were completely resistant to all isolates of *P. infestans* (Fig. 1). Most of 25 day-old plants were susceptible to this pathogen, whereas 30 day-old plants were almost resistant and occasionally showed disease symptoms (data not shown). These results suggested that resistance of *N. benthamiana* against *P. infestans* is dependent on their age, and there is no fundamental difference in pathogenicity between isolates (different races) of *P. infestans*. For subsequent analyses, the interaction of 35 day-old *N. benthamiana* plants and *P. infestans* isolate PI0-1 was used unless otherwise noted.

3.2. Both SGT1- and HSP90-mediated resistance are essential for resistance of N. benthamiana to P. infestans

Age-related resistance has been reported in various plant–microbe interactions [54,55]. Increased resistance of Arabidopsis against *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*, and tobacco against *Phytophthora parasitica* and TMV, and, has been observed in the flowering stage [56–58]. Mature tomato plants harboring either *R*-gene *Cf-9* or *Cf-9B* were resistant to *Cladosporium fulvum* with the corresponding *Avr* genes, while only *Cf-9* was effective for seedling resistance [59]. *Xa21*-dependent resistance of rice against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* gradually increased from the susceptible leaf 2 stage through to the resistant leaf 9/10 stage [60]. Downy mildew pathogen *H. arabidopsidis* isolate Emco5 displayed full virulence on cotyledons of Arabidopsis ecotype Col-0, but showed weak virulence on true leaves of Arabidopsis harboring a single locus *RPP31* [61]. These reports suggest that, at least in some interactions, mechanisms similar to R protein-

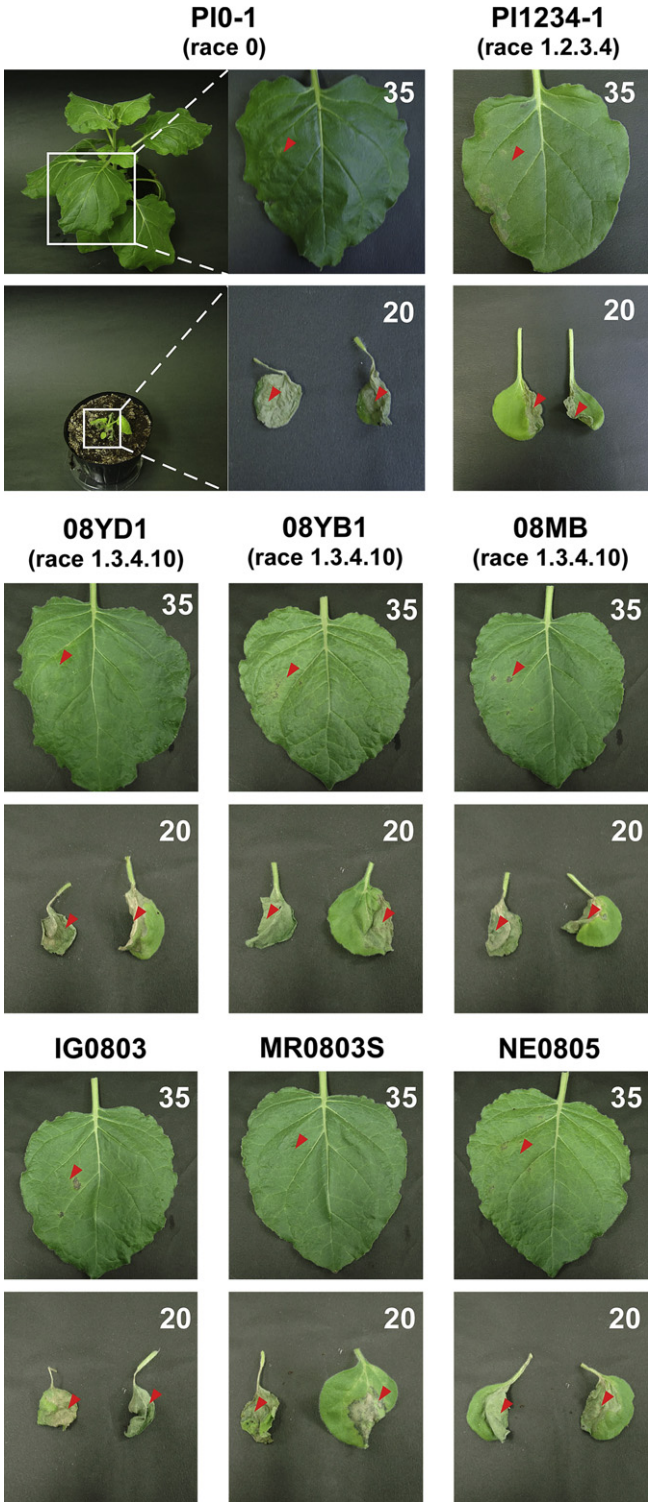

Fig. 1. Age-related resistance of *N. benthamiana* against *P. infestans*. *N. benthamiana* plants of different ages were tested for the resistance to *P. infestans* isolates PI0-1, PI1234-1, 08YD1, 08YB1, 08MB, IG0803, MR0803S or NE0805. The numbers in each panel indicate plant age in days from the 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 the development of disease symptoms was evaluated. Results shown are representative of three separate experiments. Photographs of inoculated leaves were taken 9 days post inoculation. Arrowheads indicate the point of inoculation.

mediated recognition of pathogens are involved in age-related resistance. To investigate the genes required for the function of R proteins on resistance of *N. benthamiana* to *P. infestans*, virus-induced gene silencing (VIGS) was applied to knockdown *N. benthamiana* homologues of *Arabidopsis thaliana SGT1* [26], *RAR1* [22] and *HSP90* [28]. Gene fragments of *N. benthamiana NbSGT1*, *NbRAR1* and *NbHSP90* were isolated by RT-PCR and cloned into the vector, pTV00, for TRV (tobacco rattle virus)-mediated VIGS [40]. *NbSGT1*, *NbRAR1*, and *NbHSP90* genes showed constitutive expression in *N. benthamiana* leaves, and were induced by treatment with INF1, a secretory elicitor protein derived from *P. infestans* [9] (Supplementary Fig. 1). Expression of *NbSGT1*, *NbRAR1* or *NbHSP90* was silenced by *Agrobacterium*-mediated expression of TRV derivatives containing a fragment of the target gene (Fig. 2A). Although *NbSGT1*- and *NbRAR1*-silenced plants showed normal growth compared to control TRV-infected plants, leaves of *NbSGT1*-silenced plants showed weak curling (Fig. 2B). *NbHSP90*-silenced plants showed significant dwarfing (Fig. 2B, [29]). Within 2 days post inoculation with *P. infestans* isolate PI0-1, *NbSGT1*- and *NbHSP90*-silenced plants started to show the development of visible disease symptoms (lesions) on the inoculated area (Fig. 2C). These lesions expanded beyond the inoculated area over the period of observation. In contrast, *NbRAR1*-silenced *N. benthamiana* plants showed resistance against *P. infestans* compared to control TRV-infected plants. Extension of *P. infestans* hyphae *in planta* and dead plant cells around infection sites were visualized by staining with lactophenol–trypan blue. At 48 h post inoculation, hypersensitive response (HR)-like local death of plant epidermal and mesophyll cells were occasionally observed at infection sites of control and *NbRAR1*-silenced *N. benthamiana* plants (Fig. 2D). In *NbSGT1*- or *NbHSP90*-silenced *N. benthamiana* plants, the bulk of dead mesophyll cells were obvious around the original infection site, however, *P. infestans* hyphae near the edge of the lesions were generally not surrounded by the dead plant cells (Fig. 2D). Reduced resistance of *NbSGT1*- and *NbHSP90*-silenced plants was scored by visible development of disease symptoms (Fig. 2E and F). Both *NbSGT1*- and *NbHSP90*-silenced plants showed a significant reduction in resistance against *P. infestans*. However, the development of disease symptoms was consistently faster in *NbHSP90*-silenced plants than in *NbSGT1*-silenced plants (Fig. 2F). We obtained similar results when *NbSGT1*-, *NbHSP90*- and *NbRAR1*-silenced *N. benthamiana* plants were inoculated with *P. infestans* isolate PI1234-1 (data not shown). These results indicated that SGT1 and HSP90, but not RAR1, are required for resistance of *N. benthamiana* against *P. infestans*.

3.3. *NbSGT1*-silenced *N. benthamiana* showed reduced induction of cell death in response to the treatment of INF1

INF1, an elicitin produced by *P. infestans*, was used as an elicitor to examine the effect of gene silencing on the induction of plant cell death. Leaves of *NbSGT1*- or *NbRAR1*-silenced or control *N. benthamiana* were treated with INF1 by infiltration of elicitor solution into the intercellular spaces of leaves, and induction of cell death was scored until 7 days after the treatment. *NbHSP90*-silenced plants were excluded from the analysis because these plants were severely stunted and thus difficult to infiltrate INF1 into the leaves. Cell death induced in *NbRAR1*-silenced plants was slightly, but not considerably, reduced compared to control plants, whereas *NbSGT1*-silenced plants showed significantly delayed induction of cell death (Fig. 3, [46]). These results indicated that *NbSGT1*, but not *NbRAR1*, is required for INF1-induced hypersensitive cell death.

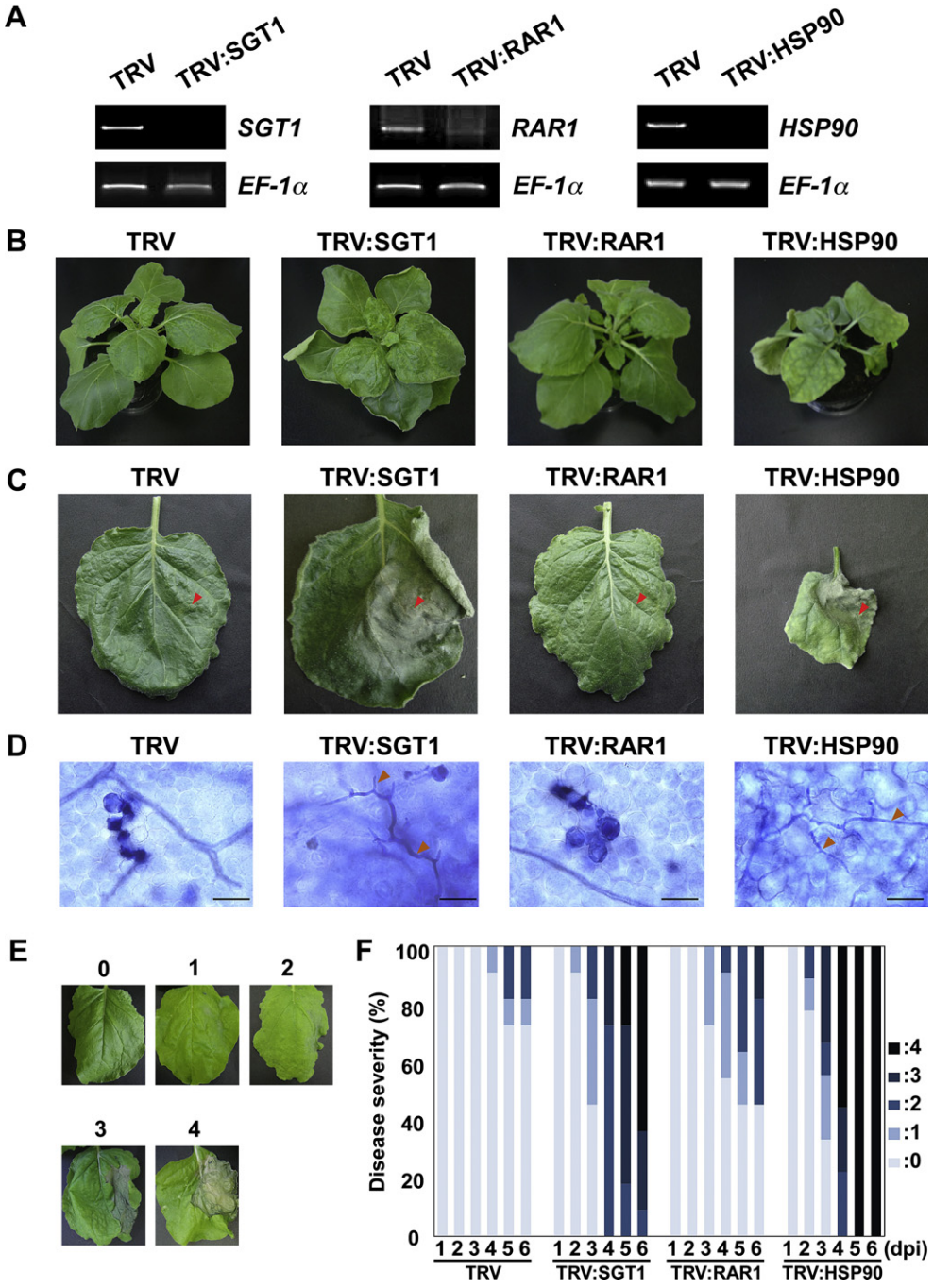

Fig. 2. Both *NbSGT1* and *NbHSP90* are essential for the resistance of *N. benthamiana* to *P. infestans*. (A) *N. benthamiana* plants were inoculated with TRV, TRV:SGT1, TRV:RAR1 or TRV:HSP90, and total RNA was isolated from *N. benthamiana* leaves. Expression of target genes was assessed by RT-PCR with gene-specific primers. Constitutively expressing *NbEF-1α* was used as an internal standard. (B) Effect of gene silencing on growth of *N. benthamiana* plants. (C) Leaves of *NbSGT1*-, *NbRAR1*- or *NbHSP90*-silenced *N. benthamiana* plants were inoculated with *P. infestans* isolate PI0-1. Arrowheads indicate the point of inoculation. Photographs were taken 9 days post inoculation (dpi). (D) Microscopic observation of *N. benthamiana* leaves 48 h post inoculation with *P. infestans*. Inoculated leaves were stained with lactophenol–trypan blue to visualize dead plant cells and hyphae of *P. infestans*. Arrowheads indicate hyphae of *P. infestans*. Bar = 50 μm. (E) Appearance of disease symptoms on leaves. Based on severity, disease symptoms on leaves were categorized into five classes (0, 1, 2, 3 and 4). (F) Plot showing percentage of *N. benthamiana* leaves with disease symptom severities represented in the five classes as shown in E, for leaves of *NbSGT1*, *NbRAR1* or *NbHSP90*-silenced plants inoculated with *P. infestans* from 1 to 6 dpi. At least 10 inoculated leaves from each silenced plant were counted.

3.4. Silencing of NbSGT1 compromised INF1-induced ROS production in N. benthamiana

Induction of ROS production is one of the common phenomena observed in various plant species in early stages of defense responses [62,63]. It has been reported that *N. benthamiana* treated with INF1 produces ROS within 12 h [52]. To investigate the effect of gene silencing on ROS production induced by INF1, *NbSGT1*- and *NbRAR1*-silenced *N. benthamiana* leaves were treated with INF1, and production of ROS (O_2^-) was measured as chemiluminescence using the luminol derivative, L-012. Twelve hours after treatment with INF1, *NbRAR1*-silenced *N. benthamiana* showed similar levels

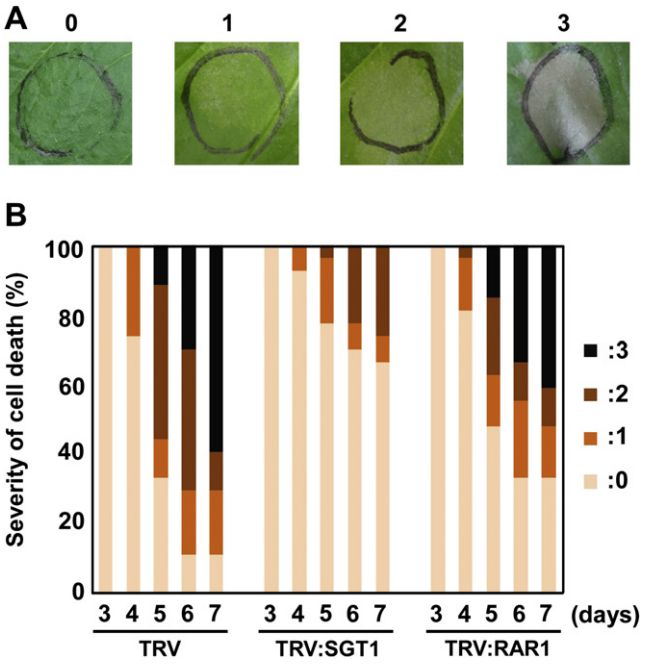

Fig. 3. *NbSGT1*-silenced *N. benthamiana* showed reduced induction of hypersensitive cell death after treatment with INF1. (A) Appearance of cell death. Based on severity, cell death was categorized into four classes (0, 1, 2 and 3). (B) *NbSGT1*- or *NbRAR1*-silenced or control *N. benthamiana* were treated with 150 nM INF1. Plot showing percentage of *N. benthamiana* leaves with cell death severities represented in the four classes as shown in A, for leaves of *NbSGT1*- or *NbRAR1*-silenced or control plants infiltrated with INF1 from 3 to 7 days after elicitor treatment. At least 24 infiltrated spots from each silenced plant were counted.

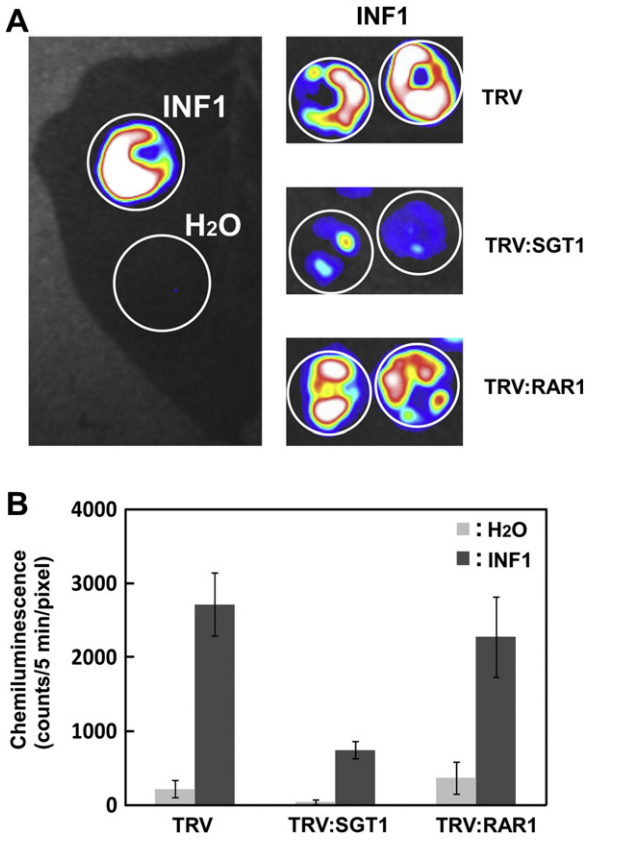

Fig. 4. Effects of gene silencing of *NbSGT1* or *NbRAR1* on INF1-induced O_2^- production. (A) Leaves of TRV-infected *N. benthamiana* were treated with H_2O or 150 nM INF1 (left panel) and gene-silenced *N. bethamiana* were treated with 150 nM INF1 (right panels). Production of O_2^- was detected as 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 ± SE from four separate experiments.

of ROS production to the control plants, while *NbSGT1*-silenced *N. benthamiana* showed significant reduction of ROS production (Fig. 4). These results indicated that *NbSGT1*, but not *NbRAR1*, is required for INF1-induced ROS production in *N. benthamiana*.

3.5. NbEDS1 and NbNDR1 are not essential for resistance of N. benthamiana to P. infestans

SGT1, RAR1 and HSP90 are reported to be required for the function of a number of R proteins from various plant species [35]. Given that *NbSGT1* and *NbHSP90* are essential for non-host resistance of *N. benthamiana* against *P. infestans*, *N. benthamiana* probably employs a mechanism similar to R proteins-mediated disease resistance against *P. infestans*. A major group of R protein identified from plants is the NB-LRR class R proteins (NLR), which are subdivided into TIR-NB-LRR and CC-NB-LRR subclasses based on the structure of N-terminal variable region. Cloned *R* genes for resistance to *P. infestans* from *Solanum* species so far encode the CC-NB-LRR class of R proteins, including race-specific R1 and R3a, and broad-spectrum Rpi-blb1 and Rpi-blb2 [3–5,64,65]. It is known that the function of TIR-NB-LRR and CC-NB-LRR class of R proteins generally require signaling factor EDS1 and NDR1, respectively [37]. To estimate the class of R protein for *N. benthamiana* resistance against *P. infestans*, requirement of *NbEDS1* and *NbNDR1* for resistance was tested. Leaves of *NbEDS1*- and *NbNDR1*-silenced *N. benthamiana* were inoculated with *P. infestans*. However, *NbEDS1*- and *NbNDR1*-silenced plants did not show any development of disease symptoms over 9 days of observation post inoculation (Supplementary Fig. 2). This result indicated that neither *NbEDS1* nor *NbNDR1* is essential for *N. benthamiana* resistance against *P. infestans*.

3.6. Expression of defense-related genes in NbSGT1- and NbRAR1-silenced plants

The effect of gene silencing of *NbSGT1* or *NbRAR1* on the expression of various defense-related genes was investigated. *NbSGT1*- or *NbRAR1*-silenced or control *N. benthamiana* plants were treated with INF1 and expression of *PR-1a* (a marker gene of salicylic acid (SA) induced genes), *hsr203j* (a marker gene for HR), *rbohB* (encodes a NADPH oxidase for ROS production during disease resistance), and *EAS*, (an enzyme required for the biosynthesis of capsidiol) were analyzed by RT-PCR. Silencing of either *NbSGT1* or *NbRAR1* had no effect on expression of most of the tested defense-related genes. However, expression of *EAS* gene in *NbSGT1*-silenced plant was significantly reduced compared with control TRV-infected and *NbRAR1*-silenced plants (Fig. 5).

3.7. Young and mature N. benthamiana induced similar level of HR by INF1 treatment

Previously, Kamoun et al. [41] reported that *P. infestans* with suppressed expression of the *inf1* gene was more pathogenic to *N. benthamiana*, suggesting that INF1 is the avirulence factor in the interaction between *P. infestans* and *N. benthamiana*. To examine whether the difference of sensitivity to INF1 is responsible for the age-related resistance of *N. benthamiana*, plants at different stages of growth were treated with INF1 elicitor and induction of

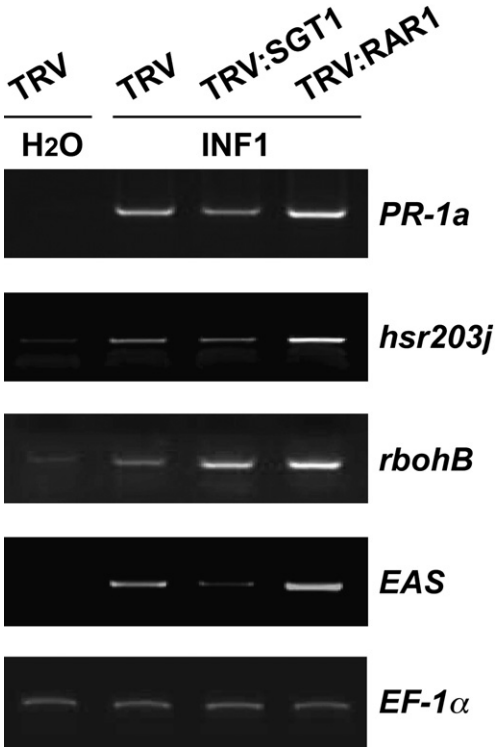

Fig. 5. Expression of defense-related genes in *NbSGT1*- or *NbRAR1*-silenced *N. benthamiana*. Total RNA was isolated from *NbSGT1*- or *NbRAR1*-silenced or control *N. benthamiana* 12 h after treatment with water (H_2O) or 150 nM INF1 (INF1). RT-PCR was performed with primers specific for *NbPR-1a*, *Nbhsr203j*, *NbrbohB* and *NbEAS1*. Constitutively expressing *NbEF-1α* was used as an internal standard.

hypersensitive cell death in young and mature plants was scored until 4 days after the treatment. Unexpectedly, comparable level of cell death was induced in both young and mature *N. benthamiana* (Fig. 6), suggested that sensitivity to INF1 is not the determinant of the age-related resistance.

In this study, we revealed that SGT1 and HSP90, components required for R protein stability, are required for the age-related "non-host" resistance of *N. benthamiana* against *P. infestans*. Multiple disease responses, including HR induction, ROS production, and expression of gene for phytoalexin production (capsidiol),

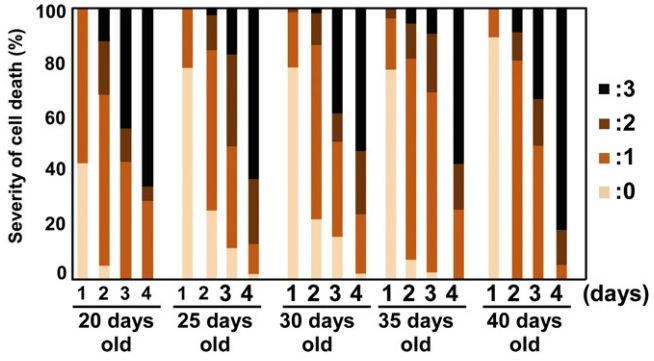

Fig. 6. Young and mature *N. benthamiana* showed comparable sensitivity to INF1. *N. benthamiana* plants of different ages were treated with 150 nM INF1. Based on severity, cell death was categorized into four classes (0, 1, 2 and 3) as shown in Fig. 3A. Plot showing percentage of *N. benthamiana* leaves with cell death severities represented in the four classes, for leaves from different age of *N. benthamiana* infiltrated with INF1 from 1 to 4 days after elicitor treatment. At least 24 infiltrated spots were counted for each age of plant.

are affected by the silencing of *SGT1* (Figs. 3–5), indicating that SGT1 is involved in early event in induction of disease resistance. It is generally considered that "non-pathogens" can not infect "non-host" plants because they do not have so-called basic compatibility as particular plant pathogen have developed mechanisms for infection to specific host plants, including penetration, suppression of plant resistance and colonization [66,67]. *SGT1*-silenced as well as young *N. benthamiana* plants inoculated with *P. infestans* showed full development of disease symptoms (Fig. 2). Moreover, *P. infestans* can produce sporangia on gene-silenced or young *N. benthamiana* under highly humid conditions [42, 43]. These results, together with presented results, indicates that *P. infestans* has basic compatibility with *N. benthamiana*, and age-related resistance of *N. benthamiana* to *P. infestans* is dependent on a mechanism similar to R protein-mediated disease resistance, perhaps the recognition of conserved molecular patterns of *P. infestans*. Champouret et al. [68] revealed that avirulence factors of *P. infestans* for the broad-spectrum resistance gene *Rpi-blb1*, Avrblb1 (ipiO), are highly conserved among *P. infestans* isolates, but a few isolates lacking ipiO can overcome *Rpi-blb1*-dependent potato resistance. This result suggests that "broad-spectrum" resistance defined by *Rpi* genes could be defeated. Although the avirulence factor(s) of *P. infestans* for *N. benthamiana* remains to be clarified, our results indicate that *N. benthamiana* could be a source of *R*-like gene(s) for broad-spectrum resistance to *P. infestans*.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.pmpp.2010.10.0010.

References

[1] Fry W. *Phytophthora infestans*: the plant (and *R* gene) destroyer. Mol Plant Pathol 2008;9:385–402.
[2] Umaerus V, Umaerus M. Inheritance of resistance to late blight. In: Bradshaw JE, Mackay GR, editors. Potato genetics. Wallingford UK: CAB International; 1994. p. 365–401.
[3] Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, et al. Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci U S A 2003;100:9128–33.
[4] van der Vossen E, Sikkema A, Hekkert B, Gros J, Stevens P, Muskens M, et al. An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant J 2003;36:867–82.
[5] van der Vossen EAG, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, et al. The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 2005;44:208–22.

[6] Kuhl JC, Hanneman Jr RE, Havey MJ. Characterization and mapping of *Rpi1*, a late-blight resistance locus from diploid (1EBN) Mexican *Solanum pinnatisectum*. Mol Genet Genomics 2001;265:977–85.
[7] Smilde WD, Brigneti G, Jagger L, Perkins S, Jones JDG. *Solanum mochiquense* chromosome IX carries a novel late blight resistance gene *Rpi-moc1*. Theor Appl Genet 2005;110:252–8.
[8] Jones DA, Takemoto D. Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules. Curr Opin Immunol 2004;16:48–62.
[9] Kamoun S, van West P, de Jong AJ, de Groot KE, Vleeshouwers VGAA, Govers F. A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. Mol Plant Microbe Interact 1997; 10:13–20.
[10] Fellbrich G, Romanski A, Varet A, Blume B, Brunner F, Engelhardt S, et al. NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*. Plant J 2002;32:375–90.
[11] Armstrong MR, Whisson SC, Pritchard L, Bos JIB, Venter E, Avrova AO, et al. An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. Proc Natl Acad Sci U S A 2005;102:7766–71.
[12] Vleeshouwers VGAA, Rietman H, Krenek P, Champouret N, Young C, Oh S-K, et al. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. PLoS One 2008;3:e2875.
[13] Kamoun S, Klucher KM, Coffey MD, Tyler BM. A gene encoding a host-specific elicitor protein of *Phytophthora parasitica*. Mol Plant Microbe Interact 1993;6:573–81.
[14] Blein J-P, Coutos-Thévenot P, Marion D, Ponchet M. From elicitins to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. Trends Plant Sci 2002;7:293–6.
[15] Takemoto D, Hardham AR, Jones DA. Differences in cell death induction by *Phytophthora elicitins* are determined by signal components downstream of MAP kinase kinase in different species of *Nicotiana* and cultivars of *Brassica rapa* and *Raphanus sativus*. Plant Physiol 2005;138:1491–504.
[16] Kamoun S, Lindqvist H, Govers F. A novel class of elicitin-like genes from *Phytophthora infestans*. Mol Plant Microbe Interact 1997;10:1028–30.
[17] Kamoun S, Hraber P, Sobral B, Nuss D, Govers F. Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. Fungal Genet Biol 1999;28:94–106.
[18] Bos JI, Kanneganti T-D, Young C, Cakir C, Huitema E, Win J, et al. The C-terminal half of *Phytophthora infestans* RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in *Nicotiana benthamiana*. Plant J 2006;48:165–76.
[19] Oh S-K, Young C, Lee M, Oliva R, Bozkurt TO, Cano LM, et al. In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. Plant Cell 2009;21:2928–47.
[20] Shirasu K, Lahaye T, Tan M-W, Zhou F, Azevedo C, Schulze-Lefert P. A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell 1999;99:355–66.
[21] Warren RF, Merritt PM, Holub E, Innes RW. Identification of three putative signal transduction genes involved in *R* gene-specified disease resistance in Arabidopsis. Genetics 1999;152:401–12.
[22] Muskett PR, Kahn K, Austin MJ, Moisan LJ, Sadanandom A, Shirasu K, et al. Arabidopsis *RAR1* exerts rate-limiting control of *R* gene-mediated defenses against multiple pathogens. Plant Cell 2002;14:979–92.
[23] Tornero P, Merritt P, Sadanandom A, Shirasu K, Innes RW, Dangl JL. *RAR1* and *NDR1* contribute quantitatively to disease resistance in Arabidopsis, and their relative contributions are dependent on the *R* gene assayed. Plant Cell 2002;14:1005–15.
[24] Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K, Schulze-Lefert P. The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science 2002;295:2073–6.
[25] Liu Y, Schiff M, Serino G, Deng X-W, Dinesh-Kumar SP. Role of SCF ubiquitin-ligase and the COP9 signalosome in the *N* gene-mediated resistance response to *Tobacco mosaic virus*. Plant Cell 2002;14:1483–96.
[26] Austin MJ, Muskett P, Kahn K, Feys BJ, Jones JDG, Parker JE. Regulatory role of *SGT1* in early *R* gene-mediated plant defenses. Science 2002;295:2077–80.
[27] Tör M, Gordon P, Cuzick A, Eulgem T, Sinapidou E, Mert-Türk F, et al. Arabidopsis SGT1b is required for defense signaling conferred by several downy mildew resistance genes. Plant Cell 2002;14:993–1003.
[28] Takahashi A, Casais C, Ichimura K, Shirasu K. HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in *Arabidopsis*. Proc Natl Acad Sci USA 2003;100:11777–82.
[29] Liu Y, Burch-Smith T, Schiff M, Feng S, Dinesh-Kumar SP. Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. J Biol Chem 2004;279:2101–8.
[30] Staal J, Kaliff M, Bohman S, Dixelius C. Transgressive segregation reveals two Arabidopsis TIR-NB-LRR resistance genes effective against *Leptosphaeria maculans*, causal agent of blackleg disease. Plant J 2006;46:218–30.
[31] Hein I, Barciszewska-Pacak M, Hrubikova K, Williamson S, Dinesen M, Soenderby IE, et al. Virus-induced gene silencing-based functional characterization of genes associated with powdery mildew resistance in barley. Plant Physiol 2005;138:2155–64.
[32] de la Fuente van Bentem S, Vossen JH, de Vries KJ, van Wees S, Tameling WIL, Dekker HL, et al. Heat shock protein 90 and its co-chaperone protein phosphatase 5 interact with distinct regions of the tomato I-2 disease resistance protein. Plant J 2005;43:284–98.
[33] Bhattarai KK, Li Q, Liu Y, Dinesh-Kumar SP, Kaloshian I. The *Mi-1*-mediated pest resistance requires *Hsp90* and *Sgt1*. Plant Physiol 2007;144:312–23.
[34] Azevedo C, Betsuyaku S, Peart J, Takahashi A, Noël L, Sadanandom A, et al. Role of SGT1 in resistance protein accumulation in plant immunity. EMBO J 2006;25:2007–16.
[35] Shirasu K. The HSP90-SGT1 chaperone complex for NLR immune sensors. Annu Rev Plant Biol 2009;60:139–64.
[36] Kadota Y, Shirasu K, Guerois R. NLR sensors meet at the SGT1-HSP90 crossroad. Trends Biochem Sci 2010;35:199–207.
[37] Dodds PN, Schwechheimer C. A breakdown in defense signaling. Plant Cell 2002;14:S5–8.
[38] Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE. Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. Proc Natl Acad Sci U S A 1998;95:10306–11.
[39] Glazebrook J. Genes controlling expression of defense responses in Arabidopsis-2001 status. Curr Opin Plant Biol 2001;4:301–8.
[40] Ratcliff F, Martin-Hernandez AM, Baulcombe DC. Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J 2001;25:237–45.
[41] Kamoun S, van West P, Vleeshouwers VGAA, de Groot KE, Govers F. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell 1998;10:1413–25.
[42] Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, et al. *Nicotiana benthamiana* gp91^{phox} homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell 2003;15:706–18.
[43] Becktell MC, Smart CD, Haney CH, Fry WE. Host–pathogen interactions between *Phytophthora infestans* and the Solanaceous hosts *Calibrachoa* × *hybridus*, *Petunia* × *hybrida*, and *Nicotiana benthamiana*. Plant Dis 2006;90: 24–32.
[44] Asai S, Ohta K, Yoshioka H. MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*. Plant Cell 2008;20:1390–406.
[45] Doke N. Prevention of the hypersensitive reaction of potato cells to infection with an incompatible race of *Phytophthora infestans* by constituents of the zoospores. Physiol Plant Pathol 1975;7:1–7.
[46] Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, et al. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc Natl Acad Sci U S A 2002;99:10865–9.
[47] Peart JR, Cook G, Feys BJ, Parker JE, Baulcombe DC. An *EDS1* orthologue is required for *N*-mediated resistance against tobacco mosaic virus. Plant J 2002;29:569–79.
[48] Schornack S, Ballvora A, Gürlebeck D, Peart J, Baulcombe D, Ganal M, et al. The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. Plant J 2004;37:46–60.
[49] Wu S, Schoenbeck MA, Greenhagen BT, Takahashi S, Lee S, Coates RM, et al. Surrogate splicing for functional analysis of sesquiterpene synthase genes. Plant Physiol 2005;138:1322–33.
[50] Cutt JR, Dixon DC, Carr JP, Klessig DF. Isolation and nucleotide sequence of cDNA clones for the pathogenesis-related proteins PR1a, PR1b and PR1c of *Nicotiana tabacum* cv. Xanthi nc induced by TMV infection. Nucleic Acids Res 1988;16:9861.
[51] Pontier D, Tronchet M, Rogowsky P, Lam E, Roby D. Activation of *hsr203*, a plant gene expressed during incompatible plant-pathogen interactions, is correlated with programmed cell death. Mol Plant Microbe Interact 1998;11:544–54.
[52] Uruma S, Shibata Y, Takemoto D, Kawakita K. *N, N*-dimethylsphingosine, an inhibitor of sphingosine kinase, induces phytoalexin production and hypersensitive cell death of Solanaceae plants without generation of reactive oxygen species. J Gen Plant Pathol 2009;74:257–66.
[53] Takemoto D, Jones DA, Hardham AR. GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens. Plant J 2003;33:775–92.
[54] Panter SN, Jones DA. Age-related resistance to plant pathogens. Adv Bot Res 2002;38:251–80.
[55] Develey-Rivière M-P, Galiana E. Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom. New Phytol 2007;175:405–16.
[56] Rusterucci C, Zhao Z, Haines K, Mellersh D. Neumann M Cameron RK. Age-related resistance to *Pseudomonas syringae* pv. *tomato* is associated with the transition to flowering in Arabidopsis and is effective against *Peronospora parasitica*. Physiol Mol Plant Pathol 2005;66:222–31.
[57] Yalpani N, Silverman P, Wilson TMA, Kleier DA, Raskin I. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 1991;3:809–18.
[58] Hugot K, Aimé S, Conrod S, Poupet A, Galiana E. Developmental regulated mechanisms affect the ability of a fungal pathogen to infect and colonize tobacco leaves. Plant J 1999;20:163–70.
[59] Panter SN, Hammond-Kosack KE, Harrison K, Jones JDG, Jones DA. Developmental control of promoter activity is not responsible for mature onset of

Cf-9B-mediated resistance to leaf mold in tomato. Mol Plant Microbe Interact 2002;15:1099–107.
[60] Century KS, Lagman RA, Adkisson M, Morlan J, Tobias R, Schwartz K, et al. Developmental control of *Xa21*-mediated disease resistance in rice. Plant J 1999;20:231–6.
[61] McDowell JM, Williams SG, Funderburg NT, Eulgem T, Dangl JL. Genetic analysis of developmentally regulated resistance to downy mildew (*Hyaloperonospora parasitica*) in *Arabidopsis thaliana*. Mol Plant Microbe Interact 2005;18:1226–34.
[62] Doke N. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol Plant Pathol 1983;23:345–57.
[63] Torres MA, Dangl JL. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 2005;8: 397–403.
[64] Ballvora A, Ercolano MR, Weiss J, Meksem K, Bormann CA, Oberhagemann P, et al. The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J 2002;30:361–71.
[65] Huang S, van der Vossen EAG, Kuang H, Vleeshouwers VGAA, Zhang N, Borm TJA, et al. Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. Plant J 2005;42:251–61.
[66] Ellingboe AH. Genetics of host–parasite interactions. In: Heitefuss R, Williams PH, editors. Physiological plant pathology (Encyclopedia of plant Physiology, New series, Vol. 4). Berlin: Springer; 1976. p. 761–78.
[67] Holub EB. Evolution of parasitic symbioses between plants and filamentous microorganisms. Curr Opin Plant Biol 2006;9:397–405.
[68] Champouret N, Bouwmeester K, Rietman H, van der Lee T, Maliepaard C, Heupink A, et al. *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato. Mol Plant Microbe Interact 2009;22: 1535–45.