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Short Communication

Isolation and characterization of an asparagine-rich protein that regulates hypersensitive cell death-mediated resistance in *Nicotiana* plants

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Abstract We isolated and characterized *Ralstonia solanacearum*-responsive genes (*RsRGs*) related to the hypersensitive response (HR) by virus-induced gene silencing using *Nicotiana benthamiana* and the potato virus X vector system. We focused on *RsRGM10*, because induction of HR was delayed in *RsRGM10*-silenced plants challenged with incompatible *R. solanacearum* 8107, which induces the HR in, *N. benthamiana*. The amino acid sequence deduced from the full-length *RsRGM10* cDNA showed approximately 90% identity with rolB overexpressed 1 (ROX1) from *N. tabacum*, and contained an asparagine-rich sequence. We designated this protein NbARP (*N. benthamiana* asparagine-rich protein). Expression of HR-related *hin1* and onset of the oxidative burst were delayed in *NbARP*-silenced plants, and induction of myelin basic kinase activity was reduced. Growth of *R. solanacearum* 8107 was accelerated in *NbARP*-silenced plants. Induction of the HR by *Pseudomonas cichorii* and *Pseudomonas syringae* pv. *syringae* was also delayed in *NbARP*-silenced plants. Silencing of *NbARP* reduced induction of HR-cell death by *Agrobacterium tumefaciens*-mediated transient expression of HR elicitors and a constitutively active form of mitogen activated protein kinase kinase. The population of *R. solanacearum* 8107 was decreased in *NbARP*-overexpressing plants. These results suggest that NbARP is closely related to the HR.

Key words: Asparagine-rich protein, Hypersensitive response, *Nicotiana benthamiana*, *Ralstonia solanacearum*.

Ralstonia solanacearum is a devastating soil-borne pathogen with a global distribution and a wide host range (Hayward 1991). In tomato, resistance to *R. solanacearum* is controlled by several loci (Thoquet et al. 1996a, b), whereas in *Arabidopsis thaliana*, it is monogenic and conferred by the *RRS1-R* gene that encodes a novel resistance protein (Lahaye 2002). Recently, PopP2, the cognate avirulence protein for *RRS1-R*, was identified and was shown to interact with the RRS1-R protein (Deslandes et al. 2003). Although there have been extensive efforts to identify resistance genes to analyze *R. solanacearum*-plant interactions, little is known about the molecular events in plants during the establishment of resistance or susceptibility to *R. solanacearum*.

Recently, we employed differential display to isolate fragments from genes that are regulated in tobacco plants inoculated with *R. solanacearum*. We identified *R. solanacearum*-responsive genes (*RsRGs*) involved in signal transduction, defense/stress responses, and cellular metabolism, as well as genes with unknown functions (Kiba et al. 2007). We have used the virus-induced gene silencing (VIGS) method to screen for genes related to the *R. solanacearum*-plant interaction using *Nicotiana benthamiana* and the potato virus X (PVX) vector system (Maimbo et al. 2007, 2010). To isolate genes related to the hypersensitive response (HR), we selected VIGS plants with compromised or

Abbreviations: DAB, 3,3′-diaminobenzidine staining; GFP, green fluorescence protein; HAI, hour after inoculation; HR, hypersensitive response; MAP kinase, mitogen activated protein kinase; MBP, myelin basic protein; NbARP, *Nicotiana benthamiana* asparagine rich protein; NtARP, *Nicotiana tabacum* asparagine rich protein; PCR, polymerase chain reaction; PVX, potato virus X; qRT-PCR, quantitative real time polymerase chain reaction; ROS, reactive oxygen species; ROX1, rolB overexpressed 1; *RsRG*, *Ralstonia solanacearum*-responsive gene; VIGS, virus-induced gene silencing.

The nucleotide sequences of *NbARP* and *NtARP* reported in this paper have been submitted to DDBJ, EMBL, and GenBank under the accession numbers AB638716 and AB638717, respectively.
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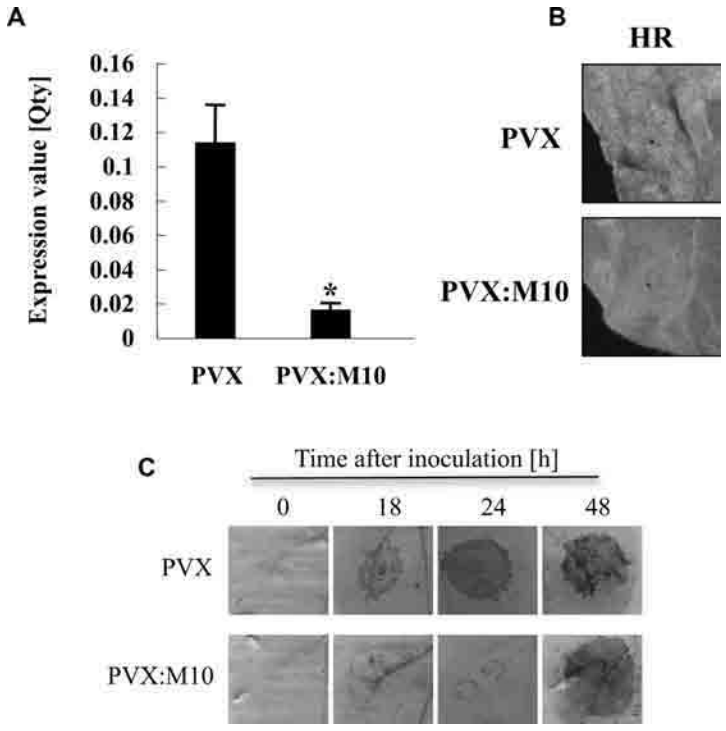

Figure 1. Effect of *RsRGM10*-silencing on HR induced by *Ralstonia solanacearum* 8107. *Nicotiana benthamiana* plants were infected with *Agrobacterium tumefaciens* GV3101 carrying either pPVX (PVX) or pPVX:M10 (PVX:M10). (A) Three weeks later, the fourth leaves above the primary *A. tumefaciens*-infected leaves were infiltrated with *R. solanacearum* 8107, and total RNA was isolated at indicated time points. Expression values of *RsRGM10* in leaves from control (PVX) and *RsRGM10*-silenced (PVX:M10) plants were estimated by qRT-PCR and are shown as [Qty] after normalization to *actin* expression. Asterisks denote values significantly different from those of control plants (* $p<0.05$, *t*-test). (B) Photographs of HR were taken 2 days after infiltration with *R. solanacearum* 8107. (C) Cell death was estimated by trypan blue staining in control (PVX) and *RsRGM10*-silenced (PVX:M10) plants leaves infiltrated with *R. solanacearum* 8107 at indicated time points (hours after inoculation).

delayed HR induction. In the present study, we selected *RsRGM10* for detailed analyses, because induction of HR was delayed in *RsRGM10*-silenced plants challenged with *R. solanacearum* 8107, which has an incompatible interaction with, and induces the HR in, *N. benthamiana*.

N. benthamiana and *Nicotiana tabacum* cv. Samsun NN were grown as described previously (Maimbo et al. 2007). *R. solanacearum* 8107, *R. solanacearum* OE1-1, *Pseudomonas cichorii* SPC9018, and *Pseudomonas syringae* pv. *syringae* MAFF03-01861R were grown for 16 h at 30°C in peptone yeast extract medium (Kiba et al. 2003; 2006). The bacterial population was measured spectrophotometrically at OD_{600}. For inoculation, the suspension was adjusted to OD_{600} of 0.1 corresponding to 10^8 cfu ml^{-1}. The leaf-infiltration method was used as the model inoculation method as described previously (Kiba et al. 2007; Maimbo et al. 2007; 2010).

The primers used in this study are listed in Supplemental Table 1. Total RNA was isolated with RNAiso Reagent (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. RNA samples were treated with DNase I (RNase-free; Takara Bio) to degrade contaminating genomic DNA. Reverse transcription was carried out with 1 μg total RNA using MMLV-reverse transcriptase (Takara Bio) with oligodT primer as described previously (Kiba et al. 2007). An internal 151-bp cDNA segment of the *RsRGM10* gene was amplified with the primers pGEMT-Pst and pGEMT-Sal. This cDNA fragment was subcloned into pGEM-T-Easy (Promega, Tokyo, Japan) to generate a new construct designated as pGEM-M10-PVX. The pGEM-M10-PVX plasmid was digested with *Pst*I and *Sal*I, and ligated into the Potato virus X vector (pPVX201) digested with *Sse*8387I and *Sal*I (Baulcombe et al. 1995). The construct containing this insert in the antisense orientation was designated as pPVX:M10. The plasmid pPVX201 with no insert (pPVX) was used as a control. These binary plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and inoculated into *N. benthamiana* leaves as described previously (Maimbo et al. 2007).

Quantitative real time polymerase chain reaction (qRT-PCR) was conducted using the method of Maimbo et al. (2007). Reverse transcription was carried out with 1 μg total RNA using the PrimeScript RT reagent kit (Takara Bio). qRT-PCR was carried out in a 20 μl reaction mixture containing 1 μl cDNA stock and 10 pM primers (*RsRGM10*, M10rtpF and M10 rtpR; *actin*, Actin-rtpF and Actin-rtpR) using the SYBR GreenER qPCR reagent system (Invitrogen, Tokyo, Japan) and

Figure 2. Deduced amino acid sequences of *NbARP*. Alignment of deduced amino acid sequences of *NbARP* from *N. benthamiana* (AB638716) with its ortholog from *N. tabacum* (*NtARP*; AB638717) and *ROX1* from *N. tabacum* (*ROX1*; DQ866831). Dots indicate identical amino acids and bars represent amino acids that are absent.

Applied Biosystems 7300 real time PCR system (Applied Biosystems, Warrington, UK). The cycling parameters were the same for all primers: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Melting curve runs were performed at the end of each PCR reaction to confirm the presence of a single product, which was an indication of primer specificity. Relative quantification of gene expression was carried out using the comparative cycle threshold [Ct] method to calculate the Qty value. All values were normalized against the expression values of the *actin* gene as an internal standard in each cDNA stock.

qRT-PCR analysis confirmed that *RsRGM10* was silenced (Figure 1A). HR was clearly observed in the control plants 24 h after inoculation (HAI) with *R. solanacearum* 8107, whereas there were no visible symptoms in similarly inoculated *RsRGM10*-silenced plants at 24 HAI (Figure 1B). Next, we confirmed the induction of cell death by trypan blue staining as described elsewhere (Torres et al. 2002). Cell death was visible from 18 HAI in the control, whereas induction of cell death was not observed in *RsRGM10*-silenced plants until 48 HAI with *R. solanacearum* 8107 (Figure 1C). These results indicated that *RsRGM10*-silenced plants showed delayed induction of cell death and the HR, suggesting that the protein encoded by *RsRGM10* is involved in inducing the HR.

To characterize *RsRGM10*, we isolated the full-length cDNA of *RsRGM10* by PCR amplification with a combination of M10-S and oligodTAD primers. The cycling parameters were as follows; 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The amplified cDNA was cloned into the pGEMT-Easy vector (pGEM-M10F). The PCR products were sequenced using M4 and RV primers with the reagents for the Big Dye Terminator Cycle Sequencing kit and Applied Biosystems 3100 Avant Automated Sequencer (Applied Biosystems, Warrington, UK). The sequence analysis was carried out using DNASIS (version 3.6; Hitachi, Yokohama, Japan) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al. 1990).

As shown in Figure 2, the amino acid sequence deduced from the full-length cDNA of *RsRGM10*, subsequently named *NbARP* (*Nicotiana benthamiana asparagine-rich protein*), contained an asparagine-rich sequence. A database search showed 99% and 90% amino acid identity with deduced amino acid sequences of its ortholog from *N. tabacum* (*NtARP*) and *rolB overexpressed 1* (*ROX1*; Cecchetti et al. 2006) from *N. tabacum*, respectively.

To further characterize the role of NbARP in HR induction, we estimated the expression of *hin1*, a marker gene for the HR (Gopalan et al. 1996) in *NbARP*-silenced plant leaves infiltrated with *R. solanacearum* 8107. Expression of *hin1* was highest in control plants at 9 HAI, but was significantly delayed in *NbARP*-silenced plants, where peak expression was observed at 12 HAI with *R. solanacearum* 8107 (Figure 3A). These results support the hypothesis that NbARP is related to induction of the HR.

Reactive oxygen species (ROS) such as hydrogen peroxide are well known signaling molecules associated with the HR (Baker and Orlandi 1995). We estimated the effect of *NbARP*-silencing on ROS production by the 3,3′-diaminobenzidine (DAB) staining staining method as described previously (Torres et al. 2002). As shown in Figure 3B, production of hydrogen peroxide occurred in

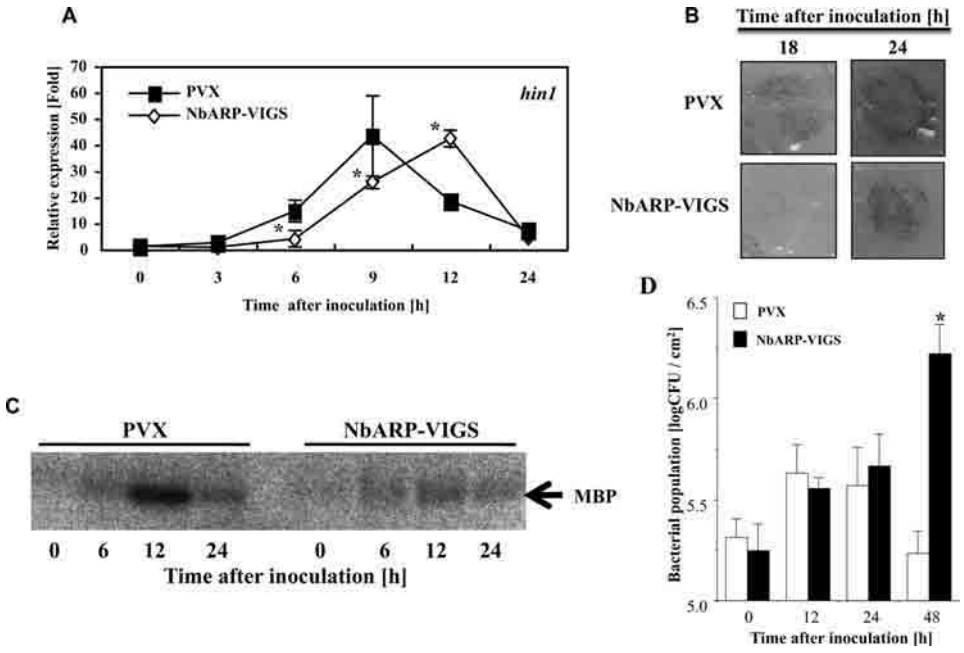

Figure 3. Phenotypic observations of *NbARP*-silenced plants challenged with *Ralstonia solanacearum* 8107. Control (PVX) and silenced (NbARP-VIGS) plant leaves were infiltrated with *R. solanacearum* 8107. (A) Total RNA was isolated at indicated time points (hours after inoculation). Relative abundance of *hin1* transcripts was analyzed by qRT-PCR using Hin1rtpF and Hin1rtpR as primers. Expression levels of *hin1* are shown relative to that in non-treated control and are normalized to *actin* expression in control (PVX) or *NbARP*-silenced (NbARP-VIGS) plants. Values are means and standard deviations from triplicate experiments. Asterisks denote values significantly different from those of control plants (* $p<0.05$ *t*-test). (B) Induction of oxidative burst by *R. solanacearum* 8107 inoculation. Hydrogen peroxide was detected by DAB staining in control (PVX) and *NbARP*-silenced (NbARP-VIGS) plants 18 and 24 HAI with *R. solanacearum* 8107. (C) Activation of MBP kinase activity by *R. solanacearum* 8107 inoculation. Total protein fractions were extracted from control (PVX) and *NbARP*-silenced (NbARP-VIGS) leaves after infiltration with *R. solanacearum* 8107 at indicated time points (hours after inoculation), and were used to determine MBP kinase activity. Arrow indicates phosphorylated MBP. (D) Effect of *NbARP*-silencing on growth of *R. solanacearum* 8107. Control (PVX) and *NbARP*-silenced (NbARP-VIGS) leaves were infiltrated with bacterial suspension of *R. solanacearum* 8107. Bacterial population was quantified by plating at specified time points (hours after inculation). Values are means of four replicate experiments with SD. Asterisks denote values significantly different from those of empty PVX controls (* $p<0.05$, *t*-test).

control plants from 18 to 24 HAI with *R. solanacearum* 8107. In contrast, there was a decrease in the production of hydrogen peroxide in *NbARP*-silenced plants at 18 and 24 HAI with *R. solanacearum* 8107, suggesting delayed induction of ROS production in *NbARP*-silenced plants.

In addition to ROS production, mitogen activated protein (MAP) kinase cascades are also signaling components related to induction of the HR (Asai and Yoshioka 2008). We analyzed myelin basic protein (MBP) kinase activity in control and *NbARP*-silenced plants challenged with *R. solanacearum* 8107. The total protein fraction was prepared by the method described previously (Maimbo et al. 2010). Briefly, leaf samples were homogenized in 10 mM Tris-HCl (pH 7.6) containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The homogenates were centrifuged at 1000 ***g*** for 10 min, and the supernatant was used as the total protein fraction. Protein concentration was determined using Quick Start Bradford Dye Reagent (Bio-Rad). The total MBP kinase assay was carried out at 25°C in 100 μl reaction buffer containing 10 μg total protein fraction, 100 μg MBP (Sigma, St. Louis, MO), 25 mM Tris-HCl (pH 8), 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, and 0.1 mM Na_3VO_4, 50 μM ATP (Sigma), and 50 μCi [γ-^{32}P]ATP (Muromachi Chemical, Kyoto, Japan) for 30 min. The reaction mixtures were separated by SDS-PAGE, and radiolabeled MBP was detected by autoradiography and visualized by GE Strom 860 and Imagequant (GE Healthcare, Tokyo, Japan). Activation of total MBP kinase was observed in control plants from 12 to 24 HAI with *R. solanacearum* 8107. In contrast, MBP activity was significantly decreased in *NbARP*-silenced plants (Figure 3C). These results suggest that NbARP may influence HR induction, in part, by regulating MAP kinase activity and oxidative burst.

To determine whether silencing of *NbARP* would affect the resistance of *N. benthamiana* to *R. solanacearum*, we estimated the size of the *R. solanacearum* 8107 population in control and *NbARP*-silenced plants. Bacterial growth was estimated by the method described previously (Shinohara et al. 2005). Leaves from control and *NbARP*-silenced plants were excised at intervals from 0 to 48 HAI with *R. solanacearum* 8107, and ground with a mortar and pestle. The original solution and its 10-fold serial dilutions were spread onto three plates of Hara-Ono medium.

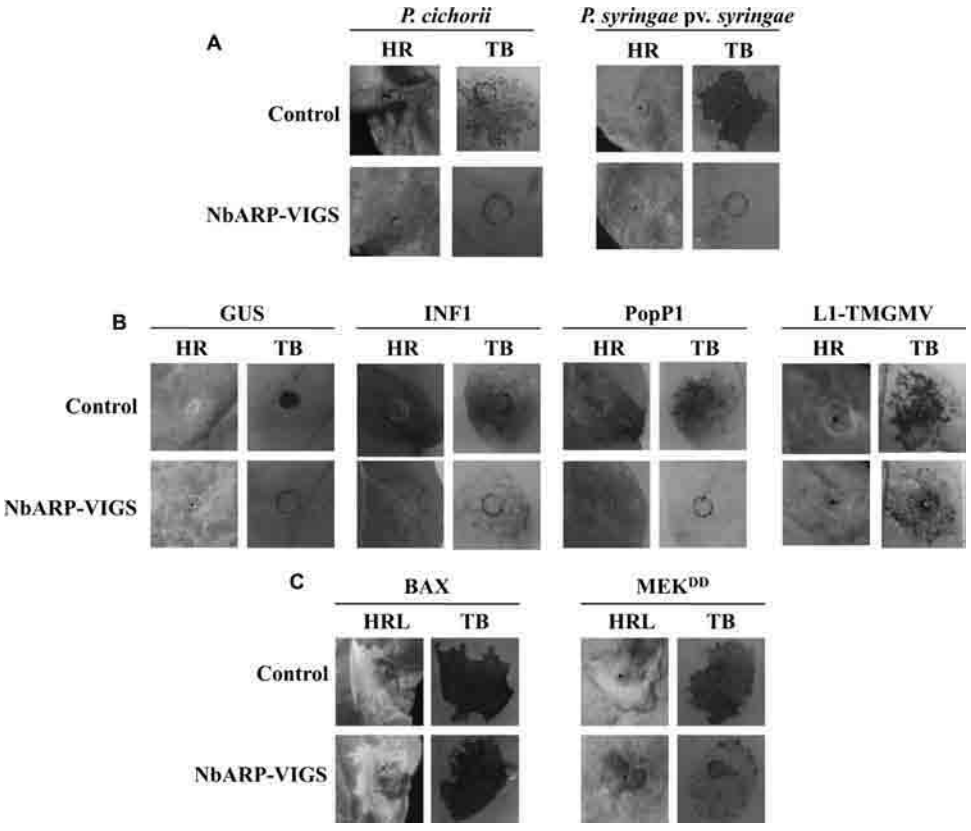

Figure 4. Effect of *NbARP*-silencing on induction of HR cell death. (A) Control (Control) and *NbARP*-silenced (NbARP-VIGS) leaves were infiltrated with *P. cichorii* or *P. syringae* pv. *syringae*. Photographs of HR-developed leaves were taken 2 days after infiltration (HR). Cell death was estimated by trypan blue staining in control (PVX) and *NbARP*-silenced (NbARP-VIGS) plants 18 HAI (hours after inoculaton) with *P. cichorii* or 12 HAI inoculation with *P. syringae* pv. *syringae*, respectively (TB). (B) Control (Control) and *NbARP*-silenced (NbARP-VIGS) leaves were infiltrated with *A. tumefaciens* harboring 35S-GUS (control GUS), 35S-INF1 (INF1), 35S-PopP1 (PopP1), or a mixture of 35S-L1 and 35S-TMGMV (L1-TMGMV). Photographs of HR development were taken 4 days after infiltration. Cell death was estimated by trypan blue staining 3 days after inoculation (TB). (C) Control (Control) and silenced (NbARP-VIGS) leaves were infiltrated with *A. tumefaciens* harboring 35S-BAX (BAX) and 35S-StMEK2^{DD} (MEK^{DD}). Photographs of HR-like lesions were taken 4 days after infiltration (HRL). Cell death was detected by trypan blue staining 3 days after infiltration (TB).

The colonies were counted after 2 days of incubation at 30°C. At 48 HAI, the population of *R. solanacearum* 8107 (in logCFU cm^{-2}) was approximately 10-fold greater in *NbARP*-silenced plants than in control plants (Figure 3D).

We further analyzed the effect of *NbARP*-silencing on induction of the HR by bacteria other than *R. solanacearum*. We used *P. cichorii* and *P. syringae* pv. *syringae*, which have incompatible reactions with, and induce HR in, *N. benthamiana*. HR developed in the control plants at 24 and 18 HAI with *P. cichorii* and *P. syringae* pv. *syringae*, respectively. In contrast, induction of the HR was significantly decreased in the *NbARP*-silenced plants. Delayed induction of cell death was also observed in *NbARP*-silenced plants challenged with *P. cichorii* and *P. syringae* pv. *syringae* (Figure 4A).

Silencing of *NbARP* resulted in decrease in the HR caused by infection with incompatible bacteria, suggesting that NbARP is related to pathogen-associated molecular patterns (PAMPs)- and effector-induced HR. Therefore, we tested the effect of *NbARP*-silencing on the HR induction by PAMPs and effectors. We used the *A. tumefaciens* system for transient expression of *PopP1*, an HR-inducible type III effector from *R. solanacearum* GMI1000 (35S-PopP1) (PopP1; Poueymiro et al. 2009), *INF1*, an HR-inducible PAMP from *Phytophthora infestans* (P35S-INF1) (INF1; Huitema et al. 2005), and for coexpression of the coat protein (CP) gene, of *Tobacco Mild Green Mosaic Virus* (35S-TMGMV-CP) and its cognate *L1* resistance gene from pepper plants (35S-L1) (TMGMV:L1; Tomita et al. 2010). The binary vector p35S-GUS containing the β-glucuronidase (GUS) gene (Katou et al. 2003) was used as a control.

As shown in Figure 4B, induction of HR cell death by INF1, PopP1, and TMGMV : L1 was observed in control plants 3 days after inoculation in control plants. On the other hand, induction of HR dell death by INF1, PopP1, and TMGM:L1 expression was decreased in *NbARP*-silenced plants. Therefore, NbARP may have a role in regulating cell death induced by biotic elicitors, such as PAMPs and effectors.

We observed decreased MBP kinase activity in

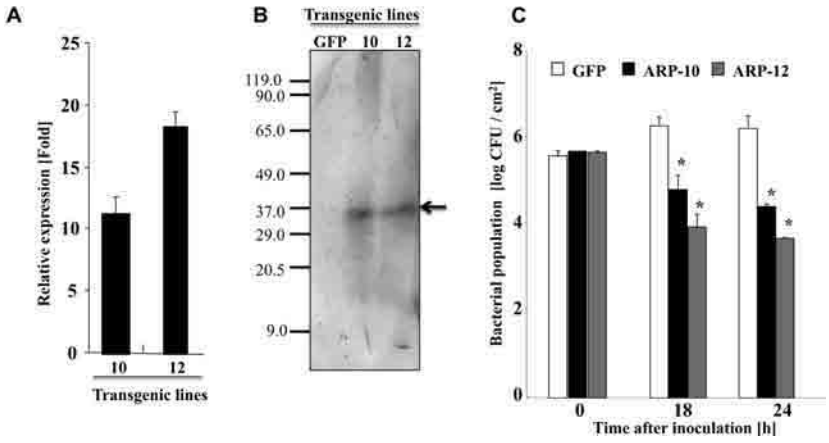

Figure 5. Phenotypic observations of *NbARP*-transgenic tobacco. (A) Total RNAs were isolated from fully expanded leaves of *GFP*-expressing control plant and two transgenic lines (ARP10 and 12). Relative expression values of total *ARP* transcripts in fully expanded tobacco leaves of T_2 *NbARP*-transformed plants (10 and 12) and *GFP*-expressing control plants were analyzed by qRT-PCR and are shown relative to that in *GFP*-expressing control. (B) Analysis of total protein fractions prepared from *GFP*-expressing control (GFP) or transgenic tobacco lines (10 and 12) by western blot using antI-FLAG tag monoclonal antibodies. Mobility of molecular weight markers shown on left. Arrow indicates immune-reactive protein bands. (C) Effect of *NbARP*-overexpression on growth of *R. solanacearum* 8107. Bacterial suspension of *R. solanacearum* 8107 was infiltrated into fully expanded leaves of *GFP*-expressing control (GFP) or transgenic tobacco lines (ARP-10 and 12). Bacterial population was quantified by plating at specified time points. Values are means of four replicate experiments with SD. Asterisks denote values significantly different from those of *GFP*-expressing controls (* $p<0.05$, *t*-test).

NbARP-silenced plants, suggesting the possible involvement of NbARP in MAP kinase-mediated HR cell death (Figure 3C). In addition to the MAP kinase cascade, the mitochondrion also integrates diverse cellular stress signals and initiates death (Lam et al. 2001). We further analyzed the possible involvement of the MAP kinase cascade and the relationship of the mitochondrial pathway with the NbARP-related HR. We used the binary vector p35S-BAX (Abramovich et al. 2003) and p35S-StMEK^{DD} (Katou et al. 2003) for transient expression analysis. Induction of HR-like lesions and cell death was observed in both control and *NbARP*-silenced plants inoculated with *A. tumefaciens* carrying p35S-BAX (Figure 4C), suggesting that there was no relationship between NbARP and mitochondria-mediated cell death. Alternatively, StMEK2^{DD} induced HR-like lesions and cell death was significantly decreased in *NbARP*-silenced plants compared with that in control plants (Figure 4C). These results suggested that NbARP may have a role in MAP kinase-mediated HR cell death.

Because we observed a reduction in the HR and resistance to *R. solanacearum* 8107 in *NbARP*-silenced plants, we tested the possibility that disease tolerant plants could be generated by overexpressing *NbARP*. We created a binary vector containing the *NbARP* gene expressed under the control of the 35S promoter. The full-length open reading frame of *NbARP* with a C-terminal FLAG tag was amplified with the primers ARPBgl-S and ARPFlag-A using pGEM-M10F as the template and cloned into pGEMT-Easy (pGEM-NbARPFlag). The pGEM-NbARPFlag was digested with *Bgl*I and *Sac*I (Takara Bio), and ligated into the pBI121 vector (CLONTECH, Tokyo, Japan) digested with the same enzymes. The construct containing the insert was designated as pBI-ARP. Tobacco plants (*N. tabacum* cv. Samsun NN) aseptically grown from seed for approximately 1 month were transformed with *NbARP* via *A. tumefaciens*-mediated leaf disc procedure (Horsch et al. 1985) and selected using 5 μg ml^{-1} bialaphos (Meiji Seika, Tokyo, Japan) as the selection reagent. We used green fluorescence protein (*GFP*)-expressing tobacco plants as the control. Total protein fraction was isolated from fully expanded leaves of *GFP*-expressing control and *NbARP*-expressing transgenic plants. The total protein fraction (10 μg) was separated by 10% SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The blots were subjected to western blot analyses with anti-Flag tag monoclonal antibody (Sigma).

To select *NbARP*-overexpressing lines, we first compared the total expression level of *ARP* in transgenic plants, which contained endogenous *NtARP* and the introduced *NbARP*, with the endogenous expression level of *NtARP* in *GFP*-expressing control plants. Two transgenic T_2 plant lines homozygous for bialaphos resistance, ARP10 and 12, showed elevated levels of total *ARP* expression compared with that in *GFP*-expressing control plants (Figure 5A). The nucleotide sequence of *NbARP* showed 99% identity with *NtARP*, and therefore, we could not distinguish *NbARP* from *NtARP* by qRT-PCR analysis. To estimate heterologous expression of *NbARP* in transgenic *N. tabacum* plants, we carried out western blot analysis with antibody raised against the Flag-tag sequence, since we could detect

only transformed Flag-tagged NbARP protein, and not endogenous NtARP protein, by western blotting. A protein with a molecular weight of approx. 37 K that showed a positive reaction with the anti-Flag antibody accumulated in both ARP10 and ARP12 plants, but not in control plants (Figure 5B). This molecular size was consistent with that estimated for the mature form of NbARP. There were no visible morphological changes in transgenic lines (data not shown). The growth of *R. solanacearum* 8107 was reduced by 100-fold in both transgenic lines at 18 and 24 HAI compared with that in the *GFP*-expressing control plants (Figure 5C).

The deduced amino acid sequence of *NbARP* showed relatively high similarity to *ROX1* from *N. tabacum*. ROX1 is believed to be involved in proliferation of procambial cells and xylem differentiation. This is because decreased levels of *ROX1* mRNA result in flowers with abnormally elongated stamens and pistils due to an increased number of cells (Cecchetti et al. 2006). As plants grow, they not only form new tissues and structures using highly coordinated cell division and cell differentiation programs, but also continuously kill many of their own cells through activation of programmed cell death. Therefore, NbARP and ROX1 might have a role in cell death regulation during HR induction and tissue differentiation, respectively.

Although the regulatory function of NbARP in inducing the HR remains obscure, our results show that there is a relationship among NbARP, MAP kinase cascades, and ROS during induction of the HR. ROS are proposed to orchestrate the establishment of plant defense responses and the HR following successful pathogen recognition (Baker and Orlandi 1995). Recently, a close linkage between MAP kinase cascades and ROS production has been reported (Yoshioka et al. 2003). Taken together, NbARP might affect MAP kinase-ROS cascade, resulting in regulated HR cell death during infection with incompatible bacteria.

Plants have evolved several types of innate immune responses to detect and respond quickly to pathogen or parasite infections (Jones and Takemoto 2004). Plants recognize PAMPs at the cell surface and establish PAMP-triggered immunity (PTI). Plants also recognize pathogen infections via polymorphic receptors that typically contain nucleotide-binding leucine-rich-repeat resistance proteins, leading to effector-triggered immunity (ETI). Both PTI and ETI are activated in incompatible interactions between plants and pathogens. Plants also possess another type of defense mechanism, basal disease resistance, which is activated by infection with virulent pathogens during compatible interactions between plants and pathogens (Jones and Dangle 2007). In this study, we observed a dramatic difference in sizes of the *R. solanacearum* 8107-population between control plants and *NbARP*-silenced plants (Figures 3D). We also confirm significant decrease of *R. solanacearum* 8107-population in *NbARP*-overexpressing transgenic plants in comparison with control plants (Figures 5C). Our data also suggested that NbARP plays a regulatory role in HR cell death induced by PAMPs and effectors (Figures 4B, C). In contrast, we did not observe any significant difference in growth of *R. solanacearum* OE1-1, which has a compatible interaction with *N. benthamiana* and *N. tabacum*, between control and *NbARP*-silenced *N. benthamiana* plants, and between *GFP*-expressing control and *NbARP*-overexpressing transgenic *N. tabacum* plants (Supplemental Figure 1). Thus, NbARP might have a role in defenses that accompany HR, including PTI and ETI, against *R. solanacearum* 8107 (incompatible interaction), but not in basal resistance against *R. solanacearum* OE1-1 (compatible interaction). In conclusion, characterization of the function of NbARP will provide novel insights into the regulatory mechanisms of the HR.

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References

Abramovitch RB, Kim YJ, Chen S, Dickman MB, Martin GB (2003) *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J* 22: 60–69

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410

Asai S, Yoshioka H (2008) The role of radical burst via MAPK signaling in plant immunity. *Plant Signal Behav* 3: 920–922

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. *Annu Rev Phytopathol* 33: 299–321

Baulcombe DC, Chapman S, Santa Cruz S (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J* 7: 1045–1053

Cecchetti V, Altamura MM, Serino G, Pomponi M, Falasca G, Costantino P, Cardarelli M (2007) *ROX1*, a gene induced by *rolB*, is involved in procambial cell proliferation and xylem differentiation in tobacco stamen. *Plant J* 49: 27–37

Deslandes L, Olivier J, Peeters N, Feng DX, Khounlotham M, Boucher C, Somssich I, Genin S, Marco Y (2003) Physical interaction between *RRS1-R*, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc Natl Acad Sci USA* 100: 8024–8029

Gopalan S, Wei W, He SY (1996) *hrp* gene-dependent induction of *hin1*: a plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. *Plant J* 10: 591–600

Hayward AC (1991) Biology and epidemiology of bacterial wilt caused by *pseudomonas solanacearum*. *Annu Rev Phytopathol* 29: 65–87

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231

Huitema E, Vleeshouwers VGAA, Cakir C, Kamoun S, Govers F (2005) Differences in intensity and specificity of hypersensitive response induction in *Nicotiana* spp. by INF1, INF2A, and INF2B of *Phytophthora infestans*. *Mol Plant Microbe Interact* 18: 183–193

Jones JD, Dangl JL (2006) The plant immune system. *Nature* 444: 323–329

Jones DA, Takemoto D (2004) Plant innate immunity—direct and indirect recognition of general and specific pathogen-associated molecules. *Curr Opin Immunol* 16: 48–62

Katou S, Yamamoto A, Yoshioka H, Kawakita K, Doke N (2003) Functional analysis of potato mitogen-activated protein kinase kinase, StMEK1. *J Gen Plant Pathol* 69: 161–168

Kiba A, Maimbo M, Kanda A, Tomiyama H, Ohnishi K, Hikichi Y (2007) Isolation and expression analysis of candidate genes related to *Ralstonia solanacearum*—tobacco interaction. *Plant Biotechnol* 24: 409–416

Kiba A, Takata O, Ohnishi K, Hikichi Y (2006) Comparative analysis of induction pattern of programmed cell death and defense-related responses during hypersensitive cell death and development of bacterial necrotic leaf spots in eggplant. *Planta* 224: 981–994

Kiba A, Tomiyama H, Takahashi H, Hamada H, Ohnishi K, Okuno T, Hikichi Y (2003) Induction of resistance and expression of defense-related genes in tobacco leaves infiltrated with *Ralstonia solanacearum*. *Plant Cell Physiol* 44: 287–295

Lahaye T (2002) The *Arabidopsis RRS1-R* disease resistance gene—uncovering the plant's nucleus as the new battlefield of plant defense? *Trends Plant Sci* 7: 425–427

Lam E, Kato N, Lawton M (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* 411: 848–853

Maimbo M, Ohnishi K, Hikichi Y, Yoshioka H, Kiba A (2007) Induction of a small heat shock protein and its functional roles in *Nicotiana* plants in the defense response against *Ralstonia solanacearum*. *Plant Physiol* 145: 1588–1599

Maimbo M, Ohnishi K, Hikichi Y, Yoshioka H, Kiba A (2010) S glycoprotein-like protein regulates defense responses in *Nicotiana* plants against *Ralstonia solanacearum*. *Plant Physiol* 152: 2023–2035

Poueymiro M, Cunnac S, Barberis P, Deslandes L, Peeters N, Cazale-Noel AC, Boucher C, Genin S (2009) Two type III secretion system effectors from *Ralstonia solanacearum* GMI1000 determine host-range specificity on tobacco. *Mol Plant Microbe Interact* 22: 538–550

Shinohara R, Kanda A, Ohnishi K, Kiba A, Hikichi Y (2005) Contribution of folate biosynthesis to *Ralstonia solanacearum* proliferation in intercellular spaces. *Appl Environ Microbiol* 71: 417–422

Thoquet P, Olivier J, Sperisen C, Rogowsky P, Laterrot H, Grimsley N (1996a) Quantitative trait loci determining resistance to bacterial wilt in tomato cultivar Hawaii 7996. *Mol Plant Microbe Interact* 9: 826–836

Thoquet P, Olivier J, Sperisen C, Rogowsky P, Prior P, Anaïs G, Mangin B, Bazin B, Nazer R, Grimsley N (1996b) Polygenic resistance of tomato plants to bacterial wilt in the French West Indies. *Mol Plant Microbe Interact* 9: 837–842

Tomita R, Sekine KT, Mizumoto H, Sakamoto M, Murai J, Kiba A, Hikichi Y, Suzuki K, Kobayashi K (2011) Genetic basis for the hierarchical interaction between *Tobamovirus* spp. and *L* resistance gene alleles from different pepper species. *Mol Plant Microbe Interact* 24: 108–117

Torres MA, Dangl JL, Jones JD (2002) Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc Natl Acad Sci USA* 99: 517–522

Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JDG, Doke N (2003) *Nicotiana benthamiana* gp91*phox* homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. *Plant Cell* 15: 706–718