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High-throughput *in planta* expression screening identifies an ADP-ribosylation factor (*ARF1*) involved in non-host resistance and *R* gene-mediated resistance

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SUMMARY

To identify positive regulators of cell death in plants, we performed a high-throughput screening, employing potato virus X-based overexpression *in planta* of a cDNA library derived from paraquat-treated *Nicotiana benthamiana* leaves. The screening of 30 000 cDNA clones enabled the identification of an ADP-ribosylation factor 1 (*ARF1*) that induces cell death when overexpressed in *N. benthamiana*. Overexpression of the guanosine diphosphate (GDP)-locked mutant of *ARF1* did not trigger cell death, suggesting that *ARF1* guanosine triphosphatase (GTPase) activity is necessary for the observed cell death-inducing activity. The *ARF1* transcript level increased strongly following treatment with *Phytophthora infestans* elicitor INF1, as well as inoculation with a non-host pathogen *Pseudomonas cichorii* in *N. benthamiana*. In addition, *ARF1* was induced in the interaction between the *N* gene and tobacco mosaic virus (TMV) in *Nicotiana tabacum*. By contrast, inoculation with the virulent pathogen *Pseudomonas syringae* pv. *tabaci* did not affect *ARF1* expression in *N. benthamiana*. Virus-induced gene silencing of *ARF1* in *N. benthamiana* resulted in a stunted phenotype, and severely hampered non-host resistance towards *P. cichorii*. In addition, *ARF1* silencing partially compromised resistance towards TMV in *N. benthamiana* containing the *N* resistance gene. By contrast, and in accordance with the *ARF1* gene expression profile, silencing of *ARF1* transcription did not alter the susceptibility of *N. benthamiana* towards the pathogen *P. syringae* pv. *tabaci*. These results strongly implicate *ARF1* in the non-host resistance to bacteria and *N* gene-mediated resistance in *N. benthamiana*.

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INTRODUCTION

Plants typically defend against a wide range of parasites by preventing them from entering attacked cells. This non-host resistance (plant immunity) is based on a two-layer protection, which includes pre- and postinvasive defence components (Lipka *et al*., 2005). On infection by host pathogens, by contrast, resistant host plants induce a complex set of signalling pathways, ultimately leading to the hypersensitive response (HR), a rapid death of invaded cells, which is associated with the restriction of pathogen growth (Heath, 2000). These host resistance processes can also occur during non-host resistance, and the extent to which these resistance responses overlap is an active area of research (for a review, see Mysore and Ryu, 2004).

High-throughput functional screening, i.e. the transient expression of a large number of genes from hosts or pathogens to elicit defence reactions, has been proven to be instrumental in the identification of regulators and components of these signalling pathways (Karrer *et al*., 1998; Nasir *et al*., 2005; Qutob *et al*., 2002; Takahashi *et al*., 2007; Takken *et al*., 2000; Torto *et al*., 2003). In this study, we performed a high-throughput screening for cell death-inducing factors, and identified an ADP-ribosylation factor 1 (*ARF1*) as a positive regulator of cell death. ARFs constitute a highly conserved family belonging to the Ras superfamily of guanosine triphosphate (GTP)-binding proteins or guanosine triphosphatases (GTPases) (Kahn *et al*., 1992). Like all members of the Ras superfamily, ARFs function as molecular switches between an 'inactive' guanosine diphosphate (GDP)-bound and an 'active' GTP-bound state. Guanine exchange factors (GEFs) convert inactive cytosolic GDP-ARF into active, membrane-associated GTP-ARF, and GTPase-activating proteins (GAPs) reform GDP-ARF (Kjeldgaard *et al*., 1996). *ARF1s* in plants are well known to play a critical role in the formation of transport vesicles by recruiting cytosolic coat proteins to sites of vesicle budding (Kirchhausen, 2000). It has been shown that *ARF1*

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specifically recruits coat protein I (COPI) to transport vesicles, thereby mediating retrograde vesicle trafficking from the Golgi to the endoplasmic reticulum (Kirchhausen, 2000). In addition to interaction with coat proteins, it has been shown in animals that *ARF1* is able to interact with other effectors to assist its role in vesicle trafficking (for a review, see Donaldson *et al.*, 2005; D'Souza-Schorey and Chavrier, 2006; Nie *et al.*, 2003; Randazzo *et al.*, 2000). Indeed, *ARF1* is known to interact with lipid-modifying enzymes, and stimulates the activity of phospholipase D (PLD; Brown *et al.*, 1993; Cockcroft *et al.*, 1994) and phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase; Jones *et al.*, 2000), leading to the production of phosphatidic acid (PA) and phosphatidylinositol 4,5-biphosphate (PIP2). It has been suggested that changes in specific phospholipids may mediate *ARF1* action in membrane traffic. Similarly, *ARF1* has been shown to regulate a dynamic pool of actin, thereby also facilitating the formation and/or dissociation of nascent vesicles from donor membranes (D'Souza-Schorey and Chavrier, 2006).

Recently, it has become clear that *ARF1* also plays a critical role in the pathogenesis of bacteria (Moss and Vaughan, 1991; Nagai *et al.*, 2002) and viruses (Richards *et al.*, 2002) in mammals. However, it remains elusive whether such a role can be attributed to *ARF1* in plants. Nomura *et al.* (2006) reported that the *Arabidopsis* ARF-GEF AtMIN7 is specifically targeted by HopM1, a *Pseudomonas syringae* virulence factor. However, to our knowledge, a direct association of *ARF1* with disease response in plants has been reported only once for a rice ARF1: RARF1 (Lee *et al.*, 2003). Although the authors did not report any loss-of-function data, they showed a rapid pathogen-induced increase in gene expression of RARF1. In addition, the expression of pathogenesis-related genes and enhanced disease resistance to a fungal pathogen was observed in transgenic *Nicotiana tabacum* plants expressing RARF1.

In this report, we provide evidence for a role of *ARF1* in disease response in *Nicotiana benthamiana*. The *ARF1* transcript level strongly increased following several types of biotic stress. In accordance with gene expression data, loss-of-function analysis of *ARF1* seriously hampered non-host resistance to *Pseudomonas cichorii* and partially compromised *N* gene-mediated resistance towards tobacco mosaic virus (TMV) in *N. benthamiana*.

RESULTS

Functional screening for plant cell death-inducing factors in *N. benthamiana* identifies an *ARF1*

The potato virus X (PVX)-based binary expression vector pSfinx (Takken *et al.*, 2000) was employed to screen for cell death-inducing genes in *N. benthamiana*. Therefore, a directional cDNA library was constructed from mRNA isolated from *N. benthamiana* leaves treated with paraquat to generate reactive oxygen species and thus to induce defence-related genes. A screening of 30 000 cDNA clones by the inoculation of *N. benthamiana* leaves allowed the identification of around 240 independent clones causing cell death within 3 weeks after inoculation. Clones, for which the cell death phenotype was subsequently confirmed by infiltration of a liquid *Agrobacterium tumefaciens* culture containing the pSfinx vector, were sequenced and analysed for gene annotation. The majority of these cDNA clones coded for proteins involved in protein degradation, such as ubiquitin-like proteins, subunits of the proteasome complex and other peptidases. Amongst others, several classes of cDNA were identified that coded for protein kinases and soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment proteins. Clone pSfinx:8-51 was investigated in more detail, as it caused cell death in *N. benthamiana* leaves as rapidly as 7 days after inoculation, as well as within 4 days after infiltration (Fig. 1). BLASTX analysis (Altschul *et al.*, 1997) of the 928-bp insert of clone pSfinx:8-51 (GENBANK accession DQ531849) revealed the presence of a full-length cDNA showing significant homology ($E = 1 \times e^{-97}$) to an *ARF1* cDNA from *Arabidopsis* (GENBANK accession NP_182239), as well as to *ARF1* cDNAs from several dicots and monocots. The predicted open reading frame (ORF) of *NbARF1* encodes a 181-amino acid protein with very high (96%) sequence identity to other known *ARF1* proteins.

Fig. 1 Overexpression of *NbARF1* causes cell death in *Nicotiana benthamiana*. *Agrobacterium tumefaciens* clones transformed with the pSfinx vector containing no insert (pSfinx, left) or the *NbARF1* cDNA (pSfinx:8-51, right) were infiltrated in the right half of the leaf of an 8-week-old *N. benthamiana* plant. A photograph of the leaf phenotype was taken 4 days after infiltration.

ARF1 is a member of a gene family and its expression is induced by challenging with INF1, TMV and the non-host pathogen *P. cichorii*

In order to characterize the genomic organization of the *ARF* genes in *N. benthamiana*, Southern hybridization was performed

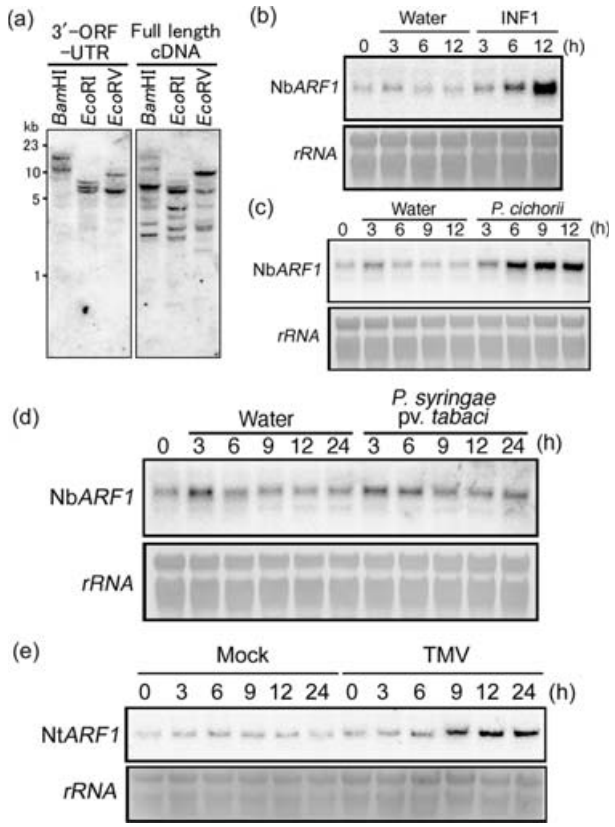

Fig. 2 Genomic organization of the *ARF1* gene and analysis of *ARF1* gene expression in *Nicotiana* spp. (a) Genomic Southern analysis suggests that *NbARF1* is a member of a small gene family in *Nicotiana benthamiana*. Ten micrograms of total DNA were digested to completion with *Bam*HI, *Eco*RI and *Eco*RV, respectively, and hybridized with the '3′-ORF-UTR' fragment (left) or full pSfinx:8-51 insert (full-length cDNA; right). (b–e) Northern analysis of *ARF1* gene expression following biotic stresses in *N. benthamiana* and *Nicotiana tabacum*. Ten micrograms of total RNA were separated in a denaturing agarose gel, and rRNAs were stained by methylene blue on the membranes as loading controls. The probe used for Northern blotting was the '3′-ORF-UTR' as used in (a). *NbARF1* gene expression was induced in *N. benthamiana* following infiltration with a solution containing 100 nM of the *Phytophthora infestans* elicitor INF1 (b), inoculation with the non-host pathogen *Pseudomonas cichorii* (OD_{600} = 0.1) (c), but not following inoculation with the virulent pathogen *Pseudomonas syringae* pv. *tabaci* (OD_{600} = 0.1) (d). The *NtARF1* transcript level increased in *N* gene-harbouring *N. tabacum* inoculated with tobacco mosaic virus following a temperature shift (e).

with the full insert sequence of pSfinx:8-51. The hybridization pattern showed several bands with diverse signal intensities, suggesting that *NbARF1* might be a member of a gene family in *N. benthamiana* (Fig. 2a, right panel). In an attempt to design a probe specific for the *ARF1* gene identified in this study, a 399-bp fragment harbouring the 3′-end of *NbARF1* ORF and the 3′-untransformed region (3′-UTR) (designated as '3′-ORF-UTR') was used as probe (Fig. 2a, left panel). The number of fragments detected by this probe was two (*Bam*HI and *Eco*RV) or three (*Eco*RI), suggesting that there is a maximum of two gene copies showing homology to this fragment. We further tested a 215-bp fragment corresponding to the *NbARF1* 3′-UTR ('3′-UTR'), but the hybridization pattern was the same as that obtained by '3′-ORF-UTR' (data not shown). To study *NbARF1* expression in relation to the defence response in *N. benthamiana*, Northern blot analysis was carried out with the probe corresponding to the *NbARF1* '3′-ORF-UTR' using RNA extracted from leaf material collected at defined time intervals after infiltration with the HR-inducing elicitor INF1 (Kamoun *et al.*, 1998), and after inoculation with the non-host bacterial pathogen *P. cichorii* (Hikichi *et al.*, 1998) or the host pathogen *P. syringae* pv. *tabaci* (Taguchi *et al.*, 2001). In addition, total RNA was extracted from *N* gene-containing *N. tabacum* leaves inoculated with TMV. A low basal *NbARF1* gene expression was detected, which was not significantly affected by water infiltration (Fig. 2b). However, on infiltration with the elicitor INF1, *NbARF1* transcription was strongly induced (Fig. 2b). Although a strong induction of *NbARF1* gene expression was detected 6 h after infiltration with *P. cichorii* (Fig. 2c), no increase in *NbARF1* expression was observed following challenge of *N. benthamiana* leaves with *P. syringae* pv. *tabaci* (Fig. 2d). To investigate whether *ARF1* expression is involved in *R* gene-mediated defence, *N. tabacum* harbouring the *N* gene was employed. The *N* gene from *N. tabacum* confers a gene-for-gene resistance towards most members of the *Tobamovirus* family by causing HR (Whitham *et al.*, 1994). We used a synchronous HR-inducing system based on a temperature shift. *Nicotiana tabacum* harbouring the *N* gene is permissive of TMV at high temperature above 26 °C (Weststeijn, 1981). Thus, incubation at 30 °C of TMV-infected, *N* gene-harbouring *N. tabacum* leaves results in the multiplication and spread of the virus without lesion formation. Shifting TMV-infected leaves from 30 °C to 20 °C induces synchronous HR formation in the infected region. We attempted a temperature shift experiment using *N* gene-harbouring *N. benthamiana* inoculated with TMV, but failed to produce synchronous HR induction. As the probe for hybridization, we used the '3-ORF-UTR' fragment of *NbARF1*. It should be noted that the *NbARF1* ORF shares 96% nucleotide sequence identity with *NtARF1* (data not shown). A slight but clear induction in *NtARF1* expression was observed after temperature shift in the leaves inoculated with TMV, but not in mock-inoculated leaves (Fig. 2e). These results suggest the involvement of *ARF1* in the *N* gene-mediated defence response towards TMV, as well as in defence reactions towards the non-host pathogen *P. cichorii*, but not towards the pathogen *P. syringae* pv. *tabaci*.

Inducible expression of *ARF1* protein triggers cell death and ion leakage

To confirm a correlation between *ARF1* protein expression and cell death, a glucocorticoid-inducible GVG expression vector

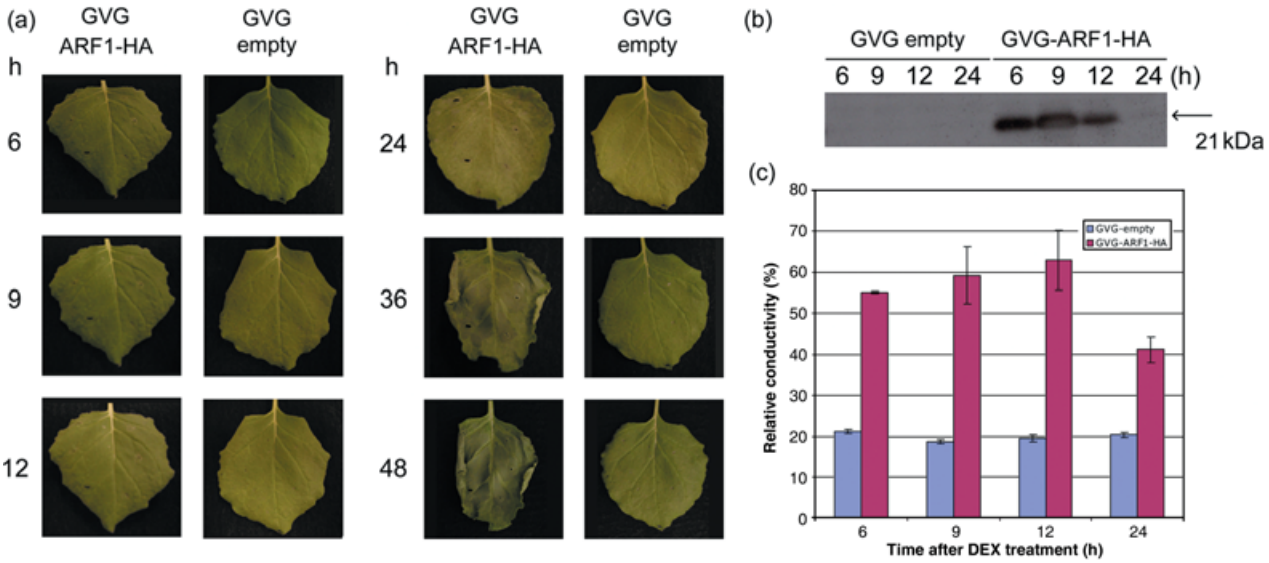

Fig. 3 Correlation between *NbARF1* protein expression and cell death in *Nicotiana benthamiana*. Leaves of an 8-week-old *N. benthamiana* plant were transiently transformed with *Agrobacterium tumefaciens* containing the empty GVG vector or GVG-ARF1-HA. Forty-eight hours later, leaves were infiltrated with 30 μM of dexamethasone (DEX) to trigger ARF1-HA protein expression. (a) Photographs of leaf phenotypes taken at different time points (6–48 h) after DEX induction. (b) Western blot analysis of ARF1-HA protein at the indicated time points (6–24 h) by employing the anti-HA antibody. (c) Ion leakage in ARF1-HA-expressing *N. benthamiana* leaves compared with control (empty GVG) leaves measured at the same time points as in (b).

(Aoyama and Chua, 1997) derivative, GVG-ARF1-HA, was constructed by cloning a fusion between the *NbARF1* ORF and a single epitope of influenza haemagglutinin (HA) into the basic GVG vector. Following transient transformation by infiltration of *Agrobacterium* harbouring an empty GVG vector or GVG-ARF1-HA, gene expression was induced by infiltrating leaves with the glucocorticoid hormone dexamethasone (DEX). Cell death occurred 24 h after DEX treatment in *ARF1*-HA-expressing plants, whereas no cell death was observed in control plants infiltrated with *Agrobacterium* harbouring empty GVG (Fig. 3a). In addition, complete desiccation of the *ARF1*-HA-expressing leaf was observed 48 h after induction (Fig. 3a). The presence of the *ARF1* protein was confirmed by immunoblot analysis using an anti-HA antibody. High quantities of *ARF1*-HA protein were detected at 6, 9 and 12 h after induction in *ARF1*-HA-transformed leaves (Fig. 3b). The attenuated expression of *ARF1*-HA detected 24 h after DEX induction can probably be explained by the fact that cell death was already advanced at that time point (Fig. 3b). These results demonstrate that overexpression of *ARF1* protein causes cell death in *N. benthamiana*. To further characterize cellular events on ARF1-HA overexpression, ion leakage was analysed. Ion leakage became clearly detectable following DEX treatment in GVG-ARF1-HA-transformed leaves, and was significantly higher than that in control leaves (Fig. 3c). Apparently, membranes became permeable on ARF1-HA overexpression.

Functional domains of *ARF1*

As described above, a common feature of small GTPases, such as *ARF1*, is the regulated binding and hydrolysis of guanine nucleotides. It is known that 'inactive' GDP-bound or 'active' GTP-bound *ARF1* mutants can be generated by specifically mutating crucial amino acid residues in the *ARF1* protein. Replacement of the threonine residue in the consensus sequence GLDAAGKT (motif P) with asparagine (T31N mutant) results in an inactive GDP-locked *ARF1* mutant. By contrast, substitution of the glutamine residue in the consensus sequence DVGGQ (motif G) by leucine (Q71L mutant) impairs intrinsic GTP hydrolysis, thereby generating a constitutively active GTP-locked *ARF1* mutant (Dascher and Balch, 1994; Kahn *et al.*, 1995; Zhang *et al.*, 1994). In addition to these two well-described mutants, another *ARF1* mutant was also obtained by deletion of the glycine residue at position 2 (G2Δ mutant), which is necessary for myristoylation of the *ARF1* protein (Franco *et al.*, 1996; Kahn *et al.*, 1995). We hypothesize that the G2Δ mutant is incapable of being tethered to plasma membrane, thereby losing its normal function. In order to investigate whether the G2, T31 and Q71 amino acid residues play a role in the cell death-inducing ability of *NbARF1*, pTA7001-derived GVG vectors were constructed containing the GDP-locked *ARF1* mutant (ARF1^{T31N}-HA), GTP-locked *ARF1* mutant (ARF1^{Q71L}-HA) and G2 deletion mutant (ARF1$^{G2\Delta}$-HA). A

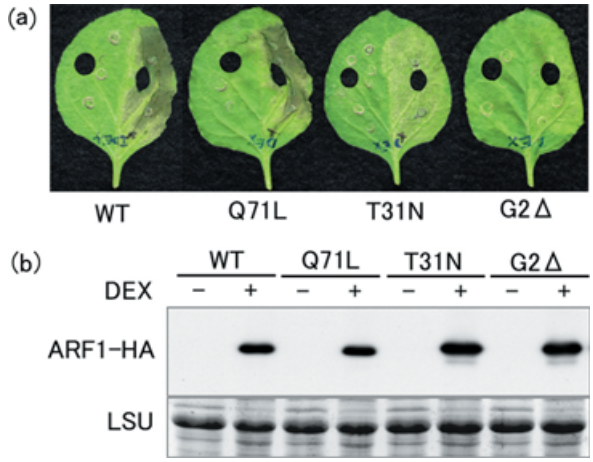

Fig. 4 Cell death caused by mutant versions of *ARF1*. Whole leaves of *Nicotiana benthamiana* were infiltrated with *agrobacterium* carrying plasmids coding for *ARF1* wild-type protein (WT) or mutated versions Q71L, T31N and G2Δ, respectively. Forty-eight hours after infiltration, the right halves of the leaves were infiltrated with dexamethasone (DEX) for the induction of protein expression. (a) Photographs were taken after another 48 h. (b) Samples for Western analysis were taken 6 h after DEX treatment. *ARF1* proteins (ARF1-HA) were detected with an anti-HA-tag antibody after the blot had been stained with amido black to visualize the large subunit of rubisCO protein (LSU) to confirm equal loading.

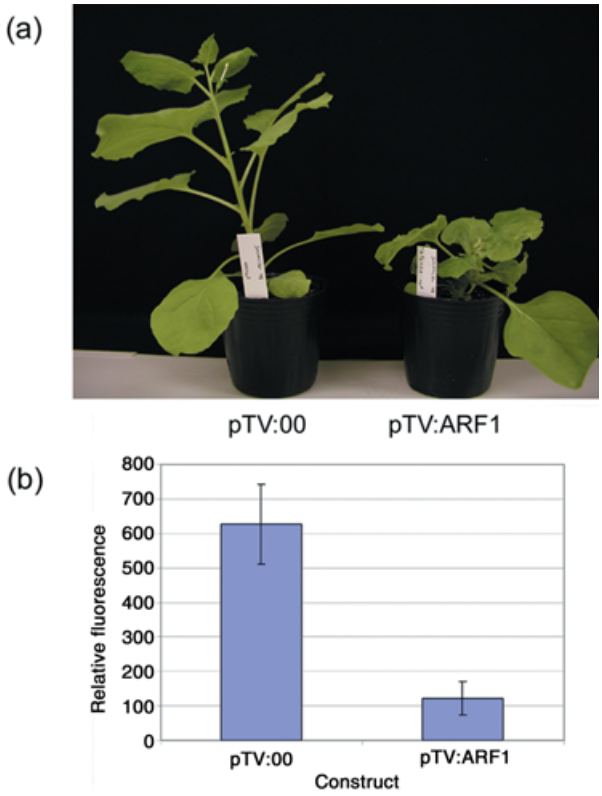

Fig. 5 Virus-induced gene silencing of *NbARF1* in *Nicotiana benthamiana*. The two youngest leaves of a 4-leaf stage *N. benthamiana* plantlet were infiltrated with pTV:ARF1 or with pTV:00 as a control treatment. Observations were done 4 weeks after inoculation. (a) Growth of *ARF1* silenced plants (pTV:*ARF1*, right) was stunted compared to control plants (pTV:00, left). (b) *NbARF1* transcript level was reduced by 80% in *ARF1* silenced plants compared to control plants as quantified by real time PCR.

pTA7001-derived vector containing the wild-type (WT) *ARF1* (ARF1^{WT}-HA) was employed as a positive control. Following transient transformation of *N. benthamiana* by infiltration with *Agrobacterium* harbouring these GVG vectors, *ARF1* gene expression was induced by infiltrating the leaves with DEX. In all cases, the proteins of the expected size were successfully expressed, as shown by Western blot analysis with anti-HA antibody (Fig. 4b). Rapid cell death occurred following DEX induction in the ARF1^{WT}-HA- and ARF1^{Q71L}-HA producing plants (Fig. 4a). No clear difference in timing and severity of cell death was found between the ARF1^{WT}-HA- and ARF1^{Q71L}-HA-overexpressing leaves. By contrast, overexpression of ARF1$^{G2\Delta}$-HA and ARF1^{T31N}-HA did not cause rapid cell death (Fig. 4a). If observations were continued for a longer time, cell death became slightly visible in leaves producing these mutants, being less severe in the G2Δ mutant than in the T31N mutant. These results suggest that GTPase activity of *ARF1* properly tethered to plasma membrane is necessary to cause rapid cell death in *N. benthamiana* leaves.

Virus-induced gene silencing of *ARF1*

Loss-of-function analysis of *ARF1* was performed by virus-induced gene silencing (VIGS). Initially, a 375-bp region from the *NbARF1* ORF was amplified and cloned in antisense orientation in a tobacco rattle virus (TRV)-derived expression vector (pTV:00; Ratcliff *et al.*, 2001). However, on infiltrating this construct into *N. benthamiana*, plant growth was arrested and the plants died within 3 weeks (results not shown), probably because overall silencing of related genes coding for ARFs occurred in these plants, thereby blocking the critical roles of *ARF1* in cell survival and development. In a second attempt, a 399-bp region was amplified from *NbARF1* comprising the full 3′UTR and 188 bp of the ORF ('3′-ORF-UTR', the same fragment as used in Fig. 2). From Southern blot analysis using this fragment (Fig. 2a), we hypothesize that, at most, two genes including *NbARF1* were targeted for silencing. When the generated pTV:ARF1 construct was infiltrated into *N. benthamiana*, plants showed a severe stunted phenotype 4 weeks later (Fig. 5a). Silencing of *NbARF1* gene expression was confirmed by real-time polymerase chain reaction (PCR) in the seventh and eighth leaves above the inoculated leaf. Transcript levels of *NbARF1* in pTV:ARF1-infiltrated plants were estimated to be reduced by 80% when compared with pTV:00 control plants (Fig. 5b). To study the effect of *ARF1* silencing on

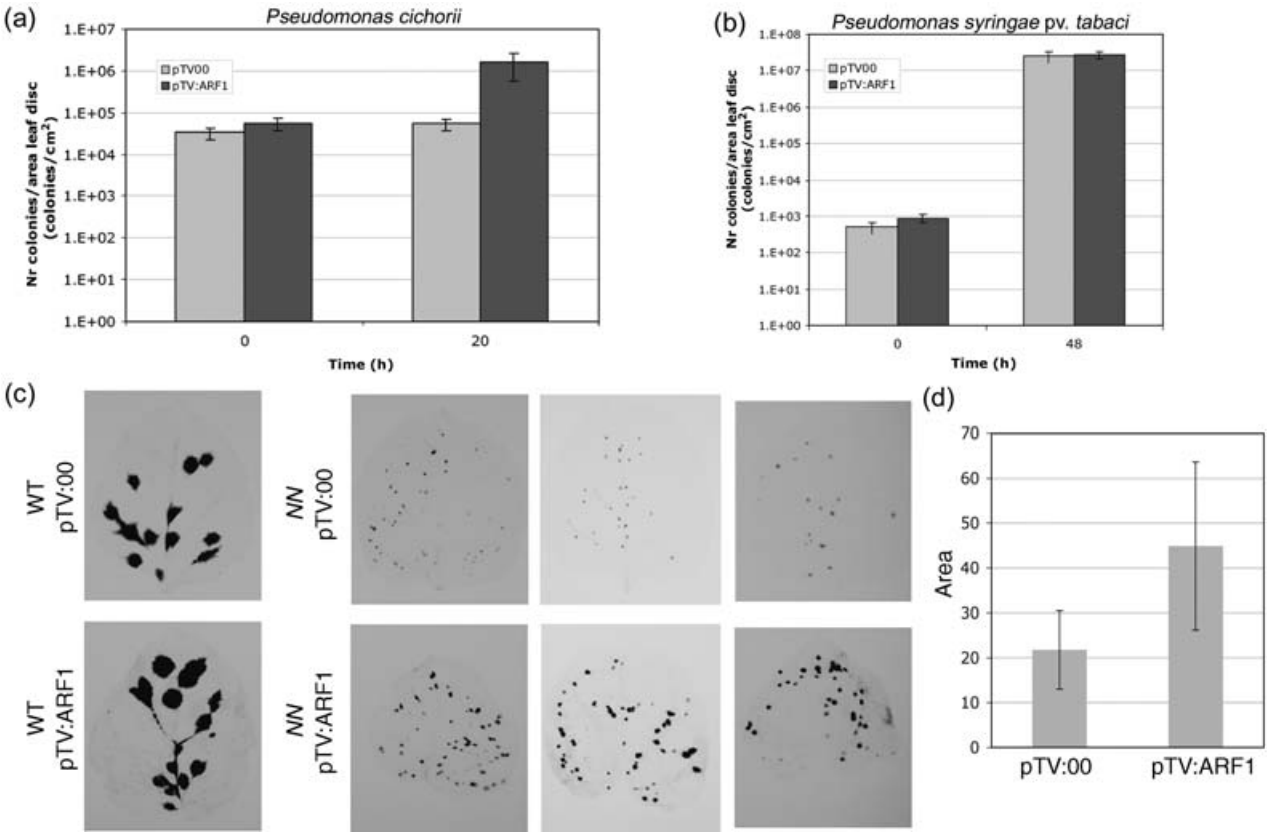

Fig. 6 Virus-induced gene silencing of *NbARF1* in *Nicotiana benthamiana* compromises defence responses. (a) *ARF1*-silenced (pTV:ARF1) or control (pTV:00) *N. benthamiana* leaves were infiltrated with *Pseudomonas cichorii* (OD_{600} = 0.01). The growth of *P. cichorii* in *ARF1*-silenced or control *N. benthamiana* leaves was determined at the indicated time points (0 and 20 h). (b) *ARF1*-silenced or control *N. benthamiana* leaves were infiltrated with *Pseudomonas syringae* pv. *tabaci* (OD_{600} = 0.0001). The growth of *P. syringae* pv. *tabaci* was determined at the indicated time points (0 and 48 h). (c) Wild-type (WT) and *N* gene-harbouring (*NN*) *N. benthamiana* plants, each inoculated with pTV:ARF1 or pTV:00, were infected with green fluorescent protein (GFP)-expressing tobacco mosaic virus (TMV, TocJ/GFP). The spread of TMV was observed in three leaves, 11 days after inoculation, by measuring GFP fluorescence, indicated as black regions. The same results were obtained in three independent experiments. (d) Bar graph showing the sizes of randomly selected spots of GFP fluorescence (n = 113 for each bar) from the leaves of control (pTV:00) and *ARF1*-silenced (pRV:ARF1) plants shown in (c). The difference is statistically significant ($P < 0.05$).

plant defence, *P. cichorii*, a non-host bacterial pathogen, was inoculated onto *N. benthamiana* leaves silenced for *ARF1*. Growth of *P. cichorii*, determined in three independent experiments 20 h after inoculation, was significantly higher in *ARF1*-silenced plants than in pTV:00-infiltrated control plants (Fig. 6a). This result suggests a crucial role for *ARF1* in non-host resistance towards a bacterial pathogen. To examine the effect of *ARF1* silencing on the defence reaction towards the host pathogen *P. syringae* pv. *tabaci*, bacterial growth was determined 48 h after infiltration in pTV:00- and pTV:ARF1-innoculated plants. However, no difference in growth was observed between these plants (Fig. 6b). These results indicate that *ARF1* silencing affects non-host resistance, but not the basal resistance to a host pathogen. To investigate the role of *ARF1* in *R* gene-mediated resistance, *N* gene-containing *N. benthamiana* was infiltrated with a green fluorescent protein (GFP)-expressing TMV (TocJ/GFP; Hori and Watanabe, 2003), which allowed the easy detection of viral spreading. As expected, very small GFP spots were observed following infection with TMV on pTV:00-infiltrated, *N* gene-containing *N. benthamiana* (Fig. 6c). However, the lesion size in *ARF1*-silenced, *N* gene-containing *N. benthamiana* was consistently larger than that in non-silenced plants in three independent experiments (Fig. 6c). The average size of randomly chosen GFP spots of *ARF1*-silenced plants was significantly ($P < 0.05$) larger than that of control plants (Fig. 6d). This result indicates a partial loss of *N* gene-mediated resistance towards TMV in *ARF1*-silenced plants.

DISCUSSION

Functional screening of plant genes identifies an ARF

The aim of this study was the identification of cell death-causing factors by functional *in vivo* screening of a cDNA library in *N. benthamiana*. To enable screening for a broad range of downstream factors involved in plant cell death, cDNA was derived from *N. benthamiana* leaves under chemically induced oxidative stress. By employing the PVX-based binary expression vector pSfinx (Takken *et al.*, 2000), we identified a cDNA encoding an *ARF1* (*NbARF1*). Members of these small GTPases are present in various eukaryotic organisms, including yeast (Stearns *et al.*, 1990), mammals (Monaco *et al.*, 1990; Tsuchiya *et al.*, 1991) and several higher plants, such as *Arabidopsis thaliana* (Regad *et al.*, 1993), rice (Higo *et al.*, 1994), potato (Szopa and Müller-Röber, 1994), maize (Verwoert *et al.*, 1995), carrot (Kiyosue and Shinozaki, 1995) and wheat (Kobayashi-Uehara *et al.*, 2001). Genomic Southern hybridization analysis suggested that several copies of *NbARF1* genes could be present in the *N. benthamiana* genome (Fig. 2a), indicating that *ARF* genes are organized in a small multigene family in plants (Higo *et al.*, 1994; Kiyosue and Shinozaki, 1995; Kobayashi-Uehara *et al.*, 2001; Regad *et al.*, 1993). We observed that transiently overexpressed GFP-tagged *NbARF1* was localized on (endo)membranes as well as in the cytosol (results not shown), corroborating previous reports (Ritzenthaler *et al.*, 2002; Takeuchi *et al.*, 2002) of the primary function of *ARF1* in vesicle trafficking in plants (Contreras *et al.*, 2004; Lee *et al.*, 2002; Memon, 2004; Molendijk *et al.*, 2004; Pimpl *et al.*, 2003). Importantly, we showed that, although the WT and GTP-locked mutant of *ARF1* caused rapid cell death, the GDP-locked mutant (ARF1^{T31N}-HA) and a mutant that could not be tethered to the plasma membrane (ARF1$^{G2\Delta}$-HA) failed to cause rapid cell death (Fig. 4). These results suggest that the GTPase function of *ARF1* is involved in the observed cell death phenotype. In addition to *NbARF1*, we identified several *ARF1*-like cDNAs that caused cell death after overexpression, although the effect was not as strong as with *NbARF1* (data not shown). This finding suggests that the enhancement of the function of *ARF1* and *ARF1*-related proteins may generally lead to cell death.

ARF1 plays a critical role in plant defence

NbARF1 transcripts were detected at low basal level (Fig. 2b), suggesting housekeeping functions for *ARF1* in *N. benthamiana*, in accordance with previous reports in plants (Kobayashi-Uehara *et al.*, 2001; Xu and Scheres, 2005). However, infiltration with the HR-inducing elicitor INF1 induced *NbARF1* (Fig. 2b). Furthermore, although *NbARF1* expression was strongly induced in *N. benthamiana* following inoculation with the non-host pathogen *P. cichorii* (Fig. 2c), no increase in *NbARF1* expression was observed following inoculation with the pathogen *P. syringae* pv. *tabaci* (Fig. 2d). This is in contrast with the induction of RARF1 observed in rice following inoculation with virulent and avirulent strains of *Magnaporthe grisea*, although the transcript level was more rapidly induced in the incompatible reaction than in the compatible interaction (Lee *et al.*, 2003). Finally, the *NtARF1* transcript level was clearly increased in *N* gene-containing *N. tabacum* inoculated with TMV (Fig. 2e).

A second observation of the role of *ARF1* in defence was demonstrated by loss-of-function analysis via VIGS. Initial attempts, employing a 375-bp region of the *NbARF1* ORF, failed, probably because silencing was targeted to most members of this conserved gene family, resulting in a phenotype which was too severe. Gebbie *et al.* (2005) similarly failed to generate homozygous *Arabidopsis* lines harbouring antisense suppression constructs for *ARF1*. VIGS of the *NbARF1* transcript level was only successful by employing a smaller region derived from the ORF, together with the full 3′UTR (3′-ORF-UTR), which resulted in a stunted phenotype (Fig. 5a). From the result of Southern blot analysis using the same 3′-ORF-UTR fragment as probe (Fig. 2a), we judged that, at most, two genes were targeted for silencing. We attempted VIGS of a single *ARF1* gene using a 215-bp fragment corresponding to the *ARF1* 3′-UTR (3′-UTR), but failed to cause gene silencing (data not shown). A stunted phenotype, as obtained here, was also observed in *Arabidopsis* following stable antisense suppression of *ARF1* (Gebbie *et al.*, 2005); this can probably be attributed to reduced cell expansion, as vesicle trafficking is crucial for the delivery of materials to the plasma membrane. In addition, reduced cell division was observed in *ARF1*-suppressed plants of *Arabidopsis* (Gebbie *et al.*, 2005), as well as in *Arabidopsis* plants containing a mutation in GNOM, a GEF of *ARF1* (Geldner *et al.*, 2003).

Silencing of *NbARF1* severely compromised the plant defence against *P. cichorii*, indicating that *ARF1* plays a role in non-host resistance (Fig. 6a). Furthermore, *R* gene-mediated resistance was partially compromised in *NbARF1*-silenced, *N* gene-containing *N. benthamiana*, as HR was delayed following inoculation with TMV (Fig. 6c,d). Although these results further support a role for *ARF1* in defence, silencing of *NbARF1* did not affect the susceptibility of *N. benthamiana* towards the virulent bacterial pathogen *P. syringae* pv. *tabaci* (Fig. 6b), indicating a differential involvement of *ARF1* in different types of defence. A specific role of *ARF1* in non-host and *R* gene-mediated resistance, but not in the basal resistance against the host pathogen, is interesting and requires future research.

To our knowledge, a direct role for *ARF1* GTPases in plant defence has been described only once for RARF1 isolated from rice. Constitutive overexpression of *RARF1* in *N. tabacum* triggered the formation of spontaneous lesions, induced pathogenesis-related genes and resulted in increased resistance towards the oomycete pathogen *Phytophthora parasitica* var. *nicotianae* (Lee *et al.*, 2003). However, the authors did not suggest a possible molecular mechanism to explain these observations. Recently, however, Nomura *et al.* (2006) described the identification of a *P. syringae*

virulence factor, HopM1, which mediated the destruction of *Arabidopsis* AtMIN7 via the host proteasome. AtMIN7 was identified as an ARF-GEF, and the authors observed that the pathogenesis of *P. syringae* in *Arabidopsis* was promoted following the destruction of AtMIN7 by HopM1. These results indicate that *P. syringae* has evolved a mechanism to eliminate a vesicle traffic pathway as an effective strategy to overcome host immunity (Nomura *et al*., 2006). The finding of Nomura *et al*. (2006) corroborates our observation. ARF-GEF is needed to convert ARF-GDP to ARF-GTP, resulting in the activation of ARF function. Therefore, it is reasonable that the loss of function of ARF-GEF and ARF both result in a decrease in plant resistance.

In addition to ARF GTPases, another family of GTPases belonging to the Ras superfamily of GTPases, namely the Rho GTPases, has recently been shown to play a key role in disease resistance and response to abiotic stress in plants (Agrawal *et al*., 2003; Gu *et al*., 2004). The best-characterized Rho GTPase in plants in connection with defence is OsRac1 from rice (Kawasaki *et al*., 1999, 2006; Ono *et al*., 2001; Suharsono *et al*., 2002). Similar to our results, Moeder *et al.* (2005) observed a differential role of OsRac1 in defence towards a non-host bacterial pathogen compared with a virulent bacterial pathogen. Stable overexpression of Rac1-T24N, a dominant-negative mutant of OsRac1, in *N. tabacum* severely compromised resistance towards the non-host pathogen *P. syringae* pv. *maculicola*, whereas it did not affect susceptibility towards the virulent pathogen *P. syringae* pv. *tabaci*. In addition, and further corroborating our *NbARF1* data in relation to viral defence, Moeder *et al.* (2005) observed reduced HR development following TMV infection of *N* gene-containing *N. tabacum* expressing the dominant-negative Rac1-T24N. Although it is well known in mammals that *ARF1* and Rac1 GTPases can interact with each other via an Arfaptin protein (D'Souza-Schorey *et al*., 1997; Shin and Exton, 2001; Tarricone *et al*., 2001), it remains elusive whether *ARF1* can interact with Rho GTPases in plants via an as yet unidentified protein.

In conclusion, we have provided strong evidence that *ARF1* is involved in cell death signalling, as its overexpression causes cell death. Our data further implicate a role for *ARF1* in plant defence, as the expression of *NbARF1* and *NtARF1* is induced on challenge with the non-host pathogen *P. cichorii* and TMV, respectively. Corroborating these data, non-host resistance and *N* gene-mediated resistance are compromised following the silencing of *NbARF1*.

EXPERIMENTAL PROCEDURES

Plant material and paraquat treatment

WT and *N* gene-containing *N. benthamiana*, as well as *N* gene-containing *N. tabacum*, plants were grown under glasshouse conditions at 23 °C without supplemental light. Fully developed leaves of approximately 8-week-old *N. benthamiana* plants were treated by application of a solution containing 50 μM paraquat (Sigma-Aldrich Japan, Tokyo, Japan), 10 mM phosphate buffer (pH 7.2) and 0.1% (v/v) Tween 20 with a soft paintbrush. Following 2 h in the dark, the plants were exposed to continuous light with an irradiance of 300 μE. The leaves were harvested after 3, 8 and 24 h, frozen in liquid nitrogen and used for total RNA isolation. One treated leaf was kept attached to the plant and monitored for cell death after a longer exposure to light.

cDNA library construction in pSfinx vector and functional screening

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN Japan, Tokyo, Japan). mRNA was isolated from total RNA by the mRNA Purification Kit (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). mRNA was subsequently used for double-stranded cDNA synthesis with asymmetrical *Sfi*I sites, employing the Creator SMART™ cDNA Library Construction Kit (Clontech Laboratories, Mountain View, CA, USA). Thereafter, the cDNA was cloned in sense orientation into the pSfinx vector (Takken *et al*., 2000), and ultracompetent *Escherichia coli* DH5α (TOYOBO, Osaka, Japan) cells were transformed with the recombinant vector. Cells were subsequently plated onto Luria–Bertani (LB) agar containing 50 μg/mL kanamycin to high density. After forming colonies for 12 h at 37 °C, approximately 80 000 colonies were collected and pooled for plasmid isolation (QIAprep Spin Kit, QIAGEN Japan). Plasmids were then employed to transform *Agrobacterium* strain LBA4404. More than 30 000 individual colonies were transferred from agar plates to 384-well microtitre plates filled with LB agar medium, and kept for further use. For functional screening, *A. tumefaciens* clones were transferred to 96-well microtitre plates harbouring liquid LB medium, and subsequently cultured for 48 h at 28 °C. Liquid-cultured cells were lifted by a tooth-pick, and inoculated onto *N. benthamiana* leaves as described by Takken *et al.* (2000). Up to five expanded leaves per plant were inoculated with 96 colonies per leaf. Putative positive clones that induced cell death around the inoculation site were rescreened by infiltrating a liquid culture of *A. tumefaciens* cells harbouring the corresponding pSfinx clone into a fully expanded leaf of an approximately 8-week-old *N. benthamiana* plant.

Southern blot and Northern blot analysis

For Southern analysis, total DNA was prepared from *N. benthamiana* leaves employing the DNeasy Plant Kit (QIAGEN Japan), digested with the restriction enzymes *Bam*HI, *Eco*RI and *Eco*RV, respectively, and separated by electrophoresis on a 1% (w/v) agarose gel before transfer to nylon membrane (Hybond N+, GE Healthcare). Total RNA was isolated from *N. benthamiana* and *N. tabacum* by the method of Nagy *et al.* (1988). Aliquots (10 μg each) were separated on formaldehyde–1% (w/v) agarose gels, and blotted onto membranes

(Hybond N+, GE Healthcare). Hybridizations were accomplished with the full insert sequence of the original pSfinx:8-51 clone (928 bp), the 3′-ORF-UTR fragment (399 bp) or the 3′-UTR fragment (215 bp).

These fragments were PCR amplified, and labelled using the TAKARA BcaBEST Labelling Kit (TaKaRa, Kyoto, Japan) and [^{32}P]dCTP (3000 Ci/mmol; GE Healthcare). Following hybridization, the membranes were washed under high stringency conditions [0.1 × standard saline citrate (SSC) and 0.1% (w/v) sodium dodecyl sulphate (SDS)] at 60 and 68 °C for Northern and Southern blots, respectively. Detection was performed using a BAS2000 bio-imaging auto-analyser (Fuji Photo Film, Tokyo, Japan).

Inducible expression of HA-tagged *ARF1* and its mutant versions

The coding sequence for a single HA tag and a diglycine linker (5′-GGGGGTTATCCATACGATGTTCCAGATTATGCT-3′; GGYPYDVPDYA) was fused in frame to the 3′-end of the *NbARF1* ORF by PCR. The resulting cDNA was directionally cloned in the *Xho*I and *Spe*I sites of a GVG vector, pTA7001 (Aoyama and Chua, 1997), to create GVG-ARF1-HA. This binary vector was subsequently transferred to *A. tumefaciens* GV3101 by chemical transformation. In order to establish transient transformation, *A. tumefaciens* cells were infiltrated into fully expanded leaves of approximately 8-week-old *N. benthamiana* plants. Forty-eight hours after infiltration, gene expression was induced by infiltrating the same leaves with DEX [30 μM in 0.1% (v/v) ethanol]. A series of pTA7001-derived vectors was constructed containing mutant versions of *ARF1*: a G2Δ mutant of *ARF1* (GVG-ARF1$^{G2\Delta}$-HA), T31N mutant of *ARF1* (GVG-ARF1^{T31N}-HA) and Q71L mutant of *ARF1* (GVG-ARF1^{Q71L}-HA). These were generated by overlap extension PCR (Ho *et al.*, 1989).

Immunoblot analysis

For the detection of ARF1-HA protein, leaf samples were ground under liquid nitrogen and extracted with 10 mM phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton-X100 (2 mL per gram of tissue). After centrifugation, the supernatants were used for electrophoresis in the presence of 0.4% (w/v) SDS on 12% (w/v) polyacrylamide slab gels (Laemmli, 1970), followed by electrophoretic transfer to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Billerico, MA, USA). After binding of the anti-HA antibody (Roche, Mannheim, Germany), the localization of antigens was detected by a secondary antibody (Promega, Madison, WI, USA), and visualized by chemiluminescence using the ECL system (GE Healthcare).

Construction of silencing vector and inoculation of *N. benthamiana*

A 399-bp *ARF1* cDNA fragment ('3′-ORF-UTR') was amplified by PCR from the original pSfinx plasmid harbouring *NbARF1* with the primer pair 5′-CTGTGCTGCTTGTTTTTGCT-3′ and 5′-CTTCGTTTACAAATTTATG-3′, annealing between positions 470 and 868 of the *ARF1* gene. The generated PCR product was then cloned in the antisense orientation into the *Kpn*I and *Spe*I sites of pTV:00 (Ratcliff *et al.*, 2001) to generate pTV:ARF1. Virus infection on *N. benthamiana* was performed as described by Ratcliff *et al.* (2001) with some minor modifications. Briefly, liquid cultures of *A. tumefaciens* GV3101 harbouring pTV:00, pTV:ARF1 or pBintra6 (Ratcliff *et al.*, 2001) were grown to saturation in liquid LB medium. Cultures were centrifuged and thereafter resuspended in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES)–KOH (pH 5.6), 10 mM $MgCl_2$ and 150 μM acetosyringone [optical density at 600 nm (OD_{600}) of 0.9 and 0.8 for pBintra6 and pTV:00/pTV:ARF1, respectively]. Cultures were subsequently incubated at room temperature for 3 h. For TRV infections, separate cultures containing pBintra6 and pTV:00 (or pTV:ARF1) were mixed in a 1 : 1 ratio. The mixture was subsequently infiltrated into the two youngest leaves of *N. benthamiana* (four-leaf stage). Four weeks after infiltration, post-transcriptional gene silencing was confirmed by real-time PCR employing a primer pair (5′-AATGACAGAGACCGTGTTGTTGA-3′ and 5′-ACAGCATCCCGAAGCTCATC-3′), annealing between positions 397 and 473 of the *NbARF1* ORF.

Inoculation with *P. cichorii* and *P. syringae* pv. *tabaci* and determination of growth kinetics

Inoculation with bacteria and determination of growth kinetics were essentially performed as described by Sharma *et al.* (2003). Briefly, a culture of *P. cichorii* SPC9001 (Hikichi *et al.*, 1998) or *P. syringae* pv. *tabaci* (Taguchi *et al.*, 2001) was grown overnight at 28 °C in liquid LB medium containing rifampicin (20 μg/mL). Following centrifugation, bacterial cells were washed and resuspended in 10 mM $MgCl_2$ (OD_{600} of 0.01 and 0.0001 for *P. cichorii* and *P. syringae* pv. *tabaci*, respectively). The bacterial cell suspension was thereafter infiltrated into *N. benthamiana* leaves using a needle-less syringe. At the indicated post-inoculation time points, small leaf discs (5 mm in diameter) were punched out of the infiltrated areas of four plants. The leaf discs were subsequently homogenized in 750 μL of 10 mM $MgCl_2$, and serial dilutions were plated onto LB plates supplemented with rifampicin. Following incubation at 28 °C for 24 h, the colonies were counted to determine the increase in the number of bacteria.

Inoculation with GFP-expressing TMV and determination of growth

The inoculation of *N. benthamiana* leaves with a GFP-expressing TMV (TocJ/GFP) was performed as described by Hori and Watanabe (2003). Eleven days after inoculation, viral growth was determined by measuring GFP fluorescence in detached inoculated leaves with a Fluorimager (Fluorimager 595, Molecular

Dynamics, Sunnyvale, CA, USA). The lesion size was calculated using APS Assess Software (http://www.apsnet.org/press/assess/).

Temperature shift experiment of TMV in *N. tabacum*

A temperature shift experiment was performed by rub-inoculating 8-week-old mature detached leaves of *N* gene-containing *N. tabacum* with TMV (10 μg/mL) in 10 mM phosphate buffer (pH 7.0) using carborandum. Mock inoculations were performed by rubbing *N. tabacum* leaves with phosphate buffer and carborandum alone. Following incubation at 30 °C for 48 h under continuous light, the temperature was reduced to 20 °C, allowing the leaves to initiate HR, which is induced on recognition of TMV. Leaf samples were taken at the indicated time points and subsequently employed for total RNA isolation.

Sequence data from this article have been deposited with the GenBank data libraries under accession number DQ531849.

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SUPPLEMENTARY MATERIAL

Figure S1. Southern blot analysis of *NbARF1* (For reviewing purpose only)

Figure S2. Northern blot analysis of *NbARF1* (For reviewing purpose only)

Figure S3. Result of RT-PCR of *NbARF1* after attempt of VIGS with *NbARF1* 3′-UTR fragment (For reviewing purpose only)

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