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Inhibitory effect on the tobacco mosaic virus infection by a plant RING finger protein

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ABSTRACT

In the yeast two-hybrid screening of plant factors interacting with tobacco mosaic virus (TMV) RNA-dependent RNA polymerase (RdRp), we found a protein containing a RING finger motif in tobacco (*Nicotiana tabacum*) and designated it as TARF (TMV-associated RING finger protein). TARF is a homologue of a *Lotus japonicus* RING finger protein (LjnsRING) involved in the symbiotic interaction between *L. japonicus* and *Mesorhizobium loti*. When *TARF* was silenced by virus-induced gene silencing (VIGS) method, TMV RNA accumulation as well as the number of foci formed by GFP-tagged TMV increased drastically. Transient overexpression of *TARF* reduced the accumulation of TMV. Moreover, *TARF* transcription was rapidly upregulated by the inoculation of TMV in tobacco plants. These results indicated that TARF is a RING finger protein that inhibits the accumulation of TMV via the interaction of TMV RdRp.

1. Introduction

Plant viruses utilize host proteins to facilitate their infection throughout the host plants while plants possess a multitude of proteins that have roles in the defense against viruses. Recent progress in genetic, biochemical and molecular biological approaches to such molecular plant–virus interactions has revealed various plant genes involved in the replication and the cell-to-cell movement of viruses as well as in the defense reactions against viruses (Ahlquist et al., 2003; Nagy, 2008; Hofmann et al., 2007; Kachroo et al., 2006). In particular, a large number of plant factors have been elucidated to positively or negatively regulate the infection of a model plant virus, *Tobacco mosaic virus* (TMV), which belongs to the genus *Tobamovirus*. TOM1 and TOM2 are membrane-bound proteins that interact with the RNA-dependent RNA polymerase (RdRp) of tomato mosaic virus (ToMV), a tobamovirus, and are required for the efficient viral replication (Yamanaka et al., 2000; Tsujimoto et al., 2003). eEF1A also interacts with the RdRp of TMV as well as the genomic RNA and promote viral multiplication (Zeenko et al., 2002; Yamaji et al., 2006, 2010). In the cell-to-cell movement process of TMV, actin and tubulin play important roles (Heinlein et al., 1995; McLean et al., 1995; Liu et al., 2005; Harries et al., 2009). A plant calcium sensor protein, SYTA, regulates endosome recycling and viral movement (Lewis and Lazarowitz, 2010). A chaperon complex including a tobacco Dna-J like protein, NtMPIP1, and a KNOX family protein, NTH201, interacts with viral MP and facilitate viral movement (Yoshii et al., 2008; Shimizu et al., 2009). In addition, *in vitro* RNA replication analysis of ToMV showed that tomato Tm-1 interacts with the RdRp of ToMV and inhibits viral replication (Ishibashi et al., 2007). Tm-1 is also involved in the determination of the non-host resistance against tobamoviruses (Ishibashi et al., 2009). Moreover, a large number of plant genes have been shown to participate in the *N*-mediated innate immune response to TMV (Soosaar et al., 2005). Thus, such approaches to uncover the molecular interaction between plants and viruses are really important to understand the molecular mechanisms of viral infection and the anti-viral strategies of plants.

The ubiquitin–proteasome pathway is a mechanism responsible for the protein turnover that regulates the majority of cellular processes (Hershko and Ciechanover, 1998). The pathway consists of sequential steps by three enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). E3 is responsible for the target specificity, because the activated and polymerized ubiquitin (polyubiquitin) is transferred to the target protein mediated by the interaction between E3 and the target. Finally, polyubiquitinated protein is translocated to 26S proteasome and degraded. There are four types of E3s, the HECT-domain containing protein, the SCF (Skp1/cullin-1/F-box) complex, the APC (anaphase promoting complex) component and RING/U-box containing protein (Citovsky et al., 2009). The genome

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of *Arabidopsis thaliana* encodes more than 1200 different components of the E3 complex (Kraft et al., 2005). The diversity of E3 complex encoded by the plant genome indicates the variety of substrate proteins targeted to proteolysis (Citovsky et al., 2009).

In plants, the ubiquitin–proteasome pathway is involved in a multitude of developmental processes, including plant hormone signaling and photosynthetic reactions (Hellmann and Estelle, 2002; Moon et al., 2004). Moreover, the ubiquitin pathway components are also involved in the plant defense reactions against pathogens including not only fungi and bacteria (Devoto et al., 2003) but also viruses (Citovsky et al., 2009). Coat proteins of TMV, barley stripe mosaic virus, brome mosaic virus, cowpea mosaic virus, cowpea severe mosaic virus and satellite panicum mosaic virus were found to be ubiquitinated (Dunigan et al., 1988; Hazelwood and Zaitlin, 1990; Jockusch and Wiegand, 2003). Movement proteins (MPs) of TMV and turnip yellow mosaic virus (TYMV) were degraded in a ubiquitin pathway-dependent manner (Reichel and Beachy, 2000; Drugeon and Jupin, 2002). TYMV RdRp was also found to be phosphorylated and ubiquitinated (Héricourt et al., 2000). The ubiquitin pathway is actually involved in the defense against viruses. The transcript level of E1 from *Nicotiana tabacum* was upregulated by the infection of TMV (Takizawa et al., 2005). CMPG1, a U-box type E3, and ACIF1, an F-box protein in SCF complex type E3, were essential for *N*-mediated hypersensitive response triggered by P50 helicase of TMV (González-Lamothe et al., 2006; Van den Burg et al., 2008). SGT1, a well-known regulator of defense responses to pathogens including viruses, interacts with SKP1, a component of SCF complex (Shirasu and Schulze-Lefert, 2003). Moreover, in the recent studies about tomato bushy stunt virus (TBSV) replication system in yeast, Nagy and colleagues found ubiquitin pathway components that affect virus RNA accumulation (Li et al., 2008; Barajas et al., 2009). Cdc34p, an E2, is included in the TBSV RNA replication complex and supports viral RNA replication, while Rps5p, a HECT-type E3, facilitates the degradation of viral RdRp and inhibits viral RNA replication. Thus the ubiquitin pathway seems to correlate with the defense against plant viruses, however the knowledge of host ubiquitin pathway that affects the infection of plant viruses is still limited.

In this study, we represent the isolation of a tobacco RING-H2 finger protein, named TARF (TMV-associated RING finger protein), which interacts with the RNA-dependent RNA polymerase (RdRp) of TMV. Silencing of *TARF* increased the accumulation of TMV and transient overexpression of TARF decreased TMV accumulation. Moreover, *TARF* transcription was induced rapidly by the inoculation of TMV. These indicate that TARF has an inhibitory role on the infection of TMV.

2. Materials and methods

2.1. Yeast two-hybrid assay

Yeast two-hybrid screening was performed using the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) as described previously by Shimizu et al. (2004) and Shimizu et al. (2009). Briefly, pBD-P, in which the P domain (nt 3492–nt 4916) of TMV RdRp was cloned into pBGKT7 (Yamaji et al., 2006), was used as a bait for screening of tobacco proteins in yeast strain AH109. Through the screening using a dropout medium and β-galactosidase assay, positive cDNA clones were selected to eliminate false-positive clones and sequenced. Database searches were performed using the BLAST algorithm at the National Center for Biotechnology Information (NCBI). 5′ rapid amplification of cDNA ends (5′ RACE) was performed to determine the 5′-teminal sequence of *TARF* using the GeneRacer kit (Invitrogen) according to the manufacturer's procedure. The cDNA sequence of TARF was deposited in GenBank under the accession number of AB551218. pBD-M, pBD-I and pBD-H were described previously by Yamaji et al. (2006). β-galactosidase activity was measured using the liquid assay as described by Shimizu et al. (2004).

2.2. Phylogenetic analysis

Phylogenetic analyses using the bootstrap option were performed using ClustalW Multiple Alignments available from the DNA Data Bank of Japan (DDBJ). Phylogenetic trees were established using the neighbor-joining method in MEGA version 3.1.

2.3. Fluorescent microscopy

The full-length cDNA of *TARF* was fused with GFP and inserted into pUC18-based 35S-driven vector to generate p35S-TARF:GFP. p35S-TARF:GFP and p35S-DsRed were introduced into tobacco BY-2 suspension culture cells by the biolistic bombardment using PDS1000/He system (Bio-Rad). BY-2 cells were incubated on Murashige–Skoog agar medium for 2 days. Confocal laser scanning microscopy was conducted with LSM5 PASCAL (Carl-Zeiss) as described previously by Yoshii et al. (2008).

2.4. VIGS

Fragments of TARFC (C-terminal fragment of TARF, nt 2845-3702), along with fragments of 5′ terminal region (NT, nt 1–584), central region (CE, nt 1525–2109) and 3′ terminal region (CT, nt 3304–3702) of *TARF* were cloned into pENTR1A, a Gateway entry vector (Invitrogen). Recombination reaction was conducted between these entry vectors and pPVX, a potato virus X (PVX)-based gateway destination vector (Yoshii et al., 2008) by Gateway LR clonase to generate pPVX-TARFC, pPVX-NT, pPVX-CE and pPVX-CT. Virus-induced gene silencing using potato virus X vector was described previously by Yoshii et al. (2008). Briefly, PVX vectors were inoculated to *Nicotiana benthamiana* using a handheld particle bombardment system (Helios Gene Gun system; Bio-Rad) according to the manufacturer's procedure. Three weeks after the bombardment, purified TMV particles or TMV-GFP (Shivprasad et al., 1999) were secondarily inoculated to the upper leaves. At appropriate days post-inoculation, leaves were sampled or observed under UV irradiation. Fluorescent images were processed by ImageJ software to calculate the number and area of fluorescent foci of TMV-GFP.

2.5. Detection of viral and mRNAs

Total RNA was extracted from leaf samples, which was subjected to Northern blot analysis or real-time RT-PCR analysis for detection of viral RNA or *TARF* mRNA. Northern blot analysis was performed as described by Yoshii et al. (2008) to detect the accumulation of *TARF* transcript using a cDNA fragment of *TARF* (nt 2356–2755) as a probe and to detect TMV RNA using a cDNA fragment of CP region (nt 5711–6187) as a probe. For the quantification of *TARF* transcript accumulation, real-time RT-PCR was performed using a primer set amplifying nt 1753–1843 of *TARF* gene. Ubiquitin mRNA was used as an internal control as described by Yoshii et al. (2008). Real-time RT-PCR reaction was conducted using SYBR Green PCR Master Mix (Applied Biosystems) by ABI PRISM 7300 (Applied Biosystems) after cDNA synthesis using High-Capacity cDNA Archive kit (Applied Biosystems) from total RNA. Real-time RT-PCR analysis of TMV RNA was performed as described by Yamaji et al. (2010). Experiments were repeated three times to calculate the mean level and standard deviation.

2.6. Agroinfiltration

TARF cDNA was cloned into a binary vector pBI121 to generate pBI-TARF. pBI121 (empty vector) and pBI-TARF were agroinfiltrated into *N. benthamiana* leaves as described by Yamaji et al. (2010). Two days post-infiltration, purified TMV particles (2 μg/ml) or TMV-GFP were mechanically inoculated into infiltrated leaves. Leaves were sampled at 2 days post-inoculation of TMV for Northern blot and real-time RT-PCR analysis or observed under UV irradiation at 5 days post-inoculation of TMV-GFP.

3. Results

3.1. Isolation of a TMV RdRp-interacting protein, TARF

The RdRp of TMV has three conserved domains, the methyltransferase (M) domain, helicase (H) domain and polymerase (P) domain. The P domain acts a crucial role in the TMV RNA replication because it has the catalytic activity of RNA polymerization (Buck, 1996). To obtain plant factors that associate with the P domain of TMV RdRp, we performed a yeast two-hybrid screening using the P domain as a bait. pBD-P that expresses the P domain fused to yeast Gal4 DNA binding domain was co-transformed with pAD-cDNA that expresses polypeptides encoded by cDNA library from *Nicotiana tabacum* cv Xanthi fused to Gal4 activation domain into yeast strain AH109 and yeast growth was evaluated on the medium lacking histidine and adenine. Positive clones selected from a total pool of 3×10^5 cDNA clones were further selected using β-galactosidase assay. Through the elimination of false-positive clones, one cDNA clone (pAD-PHF15) was identified as specifically interacting with the P domain.

5′ and 3′ RACE was performed on the pAD-PHF15 cDNA and revealed that the complete ORF of the cDNA encoded an approximately 138 kDa protein including 1233 amino acids (Fig. 1A). This protein had a six repeat of putative hemerythrin/HHE domains (aa 46–107, aa 115–176, aa 304–366, aa 372–435, aa 647–708 and aa 718–807), a putative CHY zinc finger domain (aa 988–1065) and a putative RING finger domain (aa 1102–1165). A hemerythrin/HHE domain is conserved in a family including hemerythrin, which acts as an oxygen transport protein (Karlsen et al., 2005). A RING finger domain is a typical domain conserved in E3 ubiquitin ligases. A CHY-type zinc finger domain is a domain of unknown function but is found in a family including Pirh2, a eukaryotic E3 ubiquitin ligase, which contains three distinct zinc fingers next to a RING finger domain (Leng et al., 2003). We named this protein as TARF (TMV-associated RING finger protein). The pAD-PHF15 cDNA encoded a 286-amino acid C-terminal fragment of TARF (TARFC).

The orthologous genes of TARF were only found in plant genomes indicated by database searches. The only gene functionally characterized was LjnsRING from *Lotus japonicus* (Shimomura et al., 2006), which is, interestingly, required for the successful infection and nodulation by *Mesorhizobium loti*, a symbiotic bacterium. Phylogenetic analysis based on the total amino acid sequences of TARF-orthologous proteins indicated that TARF was most closely related to an orthologue of *Solanum lycopersicum* and next to *Arabidopsis thaliana*, *L. japonicus* and *Medicago truncatula* orthologues (Fig. 1B). These results indicated that TARF is a novel tobacco RING finger protein closely related to a plant-specific RING finger protein family including LjnsRING.

To analyze the intracellular localization of TARF, we constructed vectors expressing GFP fused to both the N-terminus and the C-terminus of TARF. The vectors were introduced into tobacco BY-2 suspension culture with a vector expressing DsRed and the fluorescence was observed by confocal laser scanning microscopy. TARF:GFP was found in the nucleus, the cytoplasm and the cytoskeleton together with DsRed (Fig. 1C), indicating that TARF showed no obvious localization to particular intracellular compartment and TARF may be a soluble protein in the plant cells. The fluorescence of GFP:TARF could not be observed, maybe due to the inefficiency of expression (data not shown).

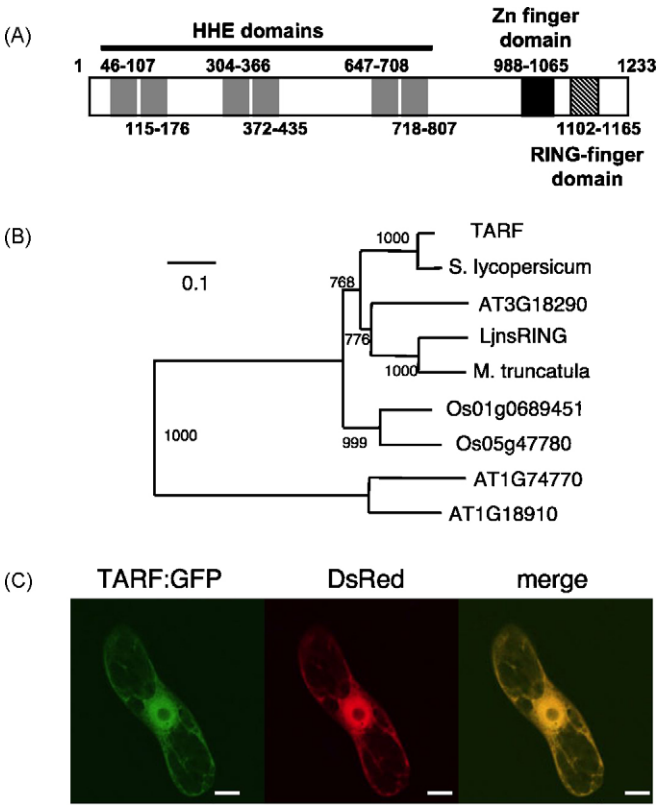

Fig. 1. Characterization of TARF. (A) Deduced structure of TARF. Hemerythrin/HHE domains, a CHY zinc finger domain and a RING finger domain are indicated as gray boxes, a closed box and a hatched box with amino acid numbers. (B) Phylogenetic analysis on TARF. Phylogenetic tree with bootstrap option based on the amino acid sequences was established on TARF-orthologous RING finger proteins using ClustalW Multiple Alignments at DNA Data Bank of Japan. The orthologous genes of TARF used in the analysis were three *Arabidopsis thaliana* genes (AT1G18910, AT1G74770 and AT3G18290), two *Oryza sativa* genes (Os01g0689451 and Os05g47780), a *Solanum lycopersicum* gene, a *Medicago truncatula* gene and a *Lotus japonicus* gene (AB272096: LjnsRING). (C) Subcellular localization of TARF:GFP. TARF:GFP and DsRed were expressed in tobacco BY-2 suspension culture cells by particle bombardment and were observed by confocal laser scanning microscopy at 2 days post-bombardment. Green fluorescence image by TARF:GFP, red fluorescence image by DsRed and the merged image are shown. Scale bars are 10 μm.

For the quantitative evaluation of the interaction between TARF and TMV RdRp in yeast cells, we conducted β-galactosidase assay. Gal4 AD vectors expressing AD fused with TARF or TARFC were co-transformed with Gal4 BD vectors expressing BD fused with domains of TMV RdRp in yeast strain Y187. The β-galactosidase activity of extracts from each yeast transformant on 2-nitrophenyl-β-D-galactopyranoside (ONPG) was measured. Yeast cells expressing TARF or TARFC with the P domain showed high levels of β-galactosidase activity (Fig. 2). However, the activities by yeast cells expressing TARF or TARFC together with the M domain, the I region or the H domain were quite low levels. These results indicated that TARF specifically interacts with the P domain of TMV RdRp via its C-terminal region.

3.2. Effect of TARF on TMV accumulation

To study the effect of TARF on the accumulation of TMV, we downregulated the transcript level of *TARF* by virus-induced gene

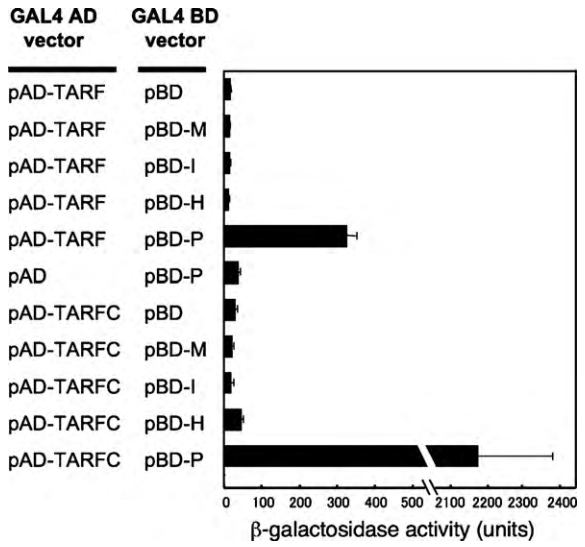

Fig. 2. Interaction between TARF and the P domain of TMV RdRp in a yeast two-hybrid assay. Gal4 AD vectors expressing AD and AD fused with TARF or TARFC were co-transformed with Gal4 BD vectors expressing BD and BD fused with domains of TMV RdRp in yeast strain Y187. Interaction was evaluated by β-galactosidase activity in a liquid assay using ONPG. The mean values of β-galactosidase activity with standard deviation in each yeast cells were indicated as units.

silencing (VIGS) method using PVX vector. *TARFC* was introduced into the multicloning site of PVX vector and the vector pPVX-TARFC was inoculated to young *N. benthamiana* plants while empty PVX vector was inoculated as a negative control. Three weeks later, upper leaves were analyzed for the confirmation of silencing. Real-time RT-PCR analysis showed that the transcript level of *TARF* significantly decreased in the PVX-TARFC infected leaves compared to that in PVX infected leaves (Fig. 3A). The growth of plants, including the height and leaf shape, was not affected by the silencing of *TARF* (data not shown). Then, TMV particles were secondarily inoculated to the *TARF*-silenced leaves and the accumulation of TMV RNA was detected by the Northern blotting (Fig. 3B). At both 1 and 2 days post-inoculation of TMV, the accumulation of TMV RNA drastically increased in *TARF*-silenced leaves compared to non-silenced leaves. Real-time RT-PCR analysis for quantitative evaluation of TMV RNA accumulation indicated that viral RNA accumulation in *TARF*-silenced leaves increased over 5 times more than that in non-silenced leaves (Fig. 3C). These results indicated that the silencing of *TARF* increases the accumulation of TMV RNA.

To visualize the spread of TMV, TMV-GFP was inoculated to silenced or non-silenced leaves and, 1 week later, the leaves were observed under UV irradiation. Fig. 3D shows the representative images of TMV-GFP foci in *TARF*-silenced and non-silenced leaves, indicating that obviously increased level of fluorescence by TMV-GFP was observed in *TARF*-silenced leaves compared to that in non-silenced leaves. Importantly, the number of TMV-GFP foci significantly increased in *TARF*-silenced leaves while the mean area of TMV-GFP foci was not apparently different (Fig. 3E and F). The results that the silencing of *TARF* affected the formation of TMV-GFP infection foci rather than the spread of TMV-GFP suggested that TARF negatively regulates TMV accumulation at an early stage in viral replication rather than viral cell-to-cell movement stage.

Since the C-terminal region of TARF including RING finger domain was relatively conserved in other closely related proteins encoded by plants compared to the N-terminal and central regions, the silencing targeting the C-terminus of TARF might repress the expression of other RING finger proteins closely related to TARF in *N. benthamiana*. Therefore, we performed PVX-mediated silencing targeting N-terminal (nt 1–584; TARF-NT) and central (nt

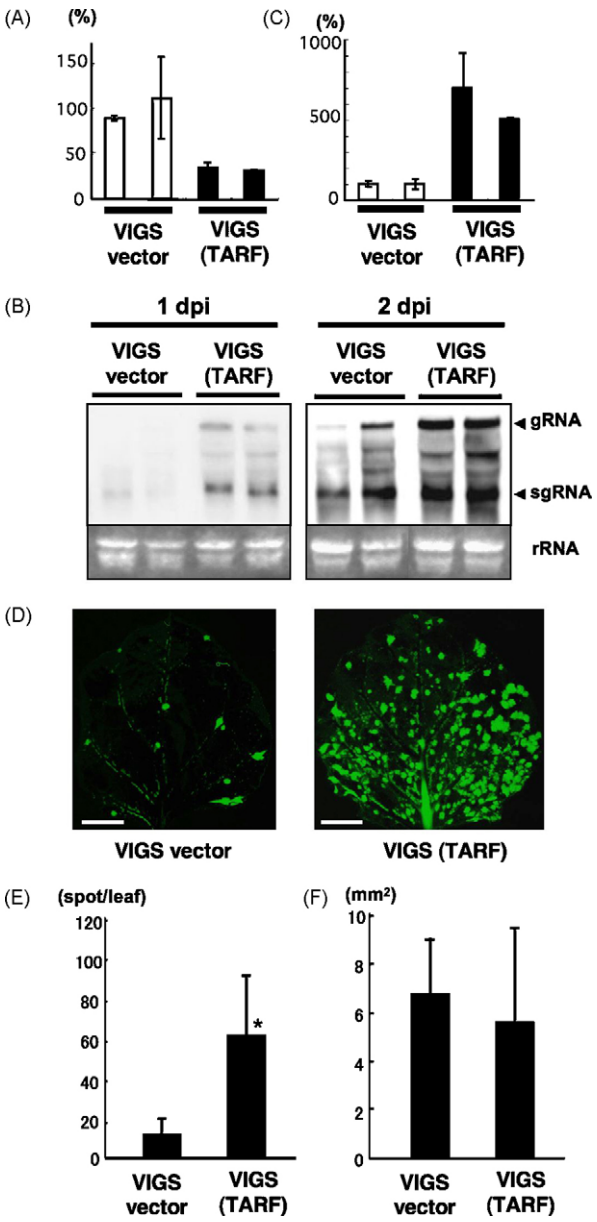

Fig. 3. TMV accumulation in *TARF*-silenced plants. (A) Silencing of *TARF*. Plants were inoculated with PVX (vector) and PVX-TARFC (TARF) to induce VIGS. Total RNA was isolated from each plants at 3 weeks post-VIGS induction and subjected to real-time RT-PCR analysis using *TARF*-specific primers. The accumulation level of ubiquitin mRNA was used as a reference. The mean level in the non-silenced leaves was taken as a standard (100%). The results from two independent experiments are shown. (B and C) TMV RNA accumulation in *TARF*-silenced plants. Purified TMV particles were inoculated to non-silenced and *TARF*-silenced plants. The accumulation of TMV RNA was analyzed by Northern blot analysis using TMV CP-specific probe at 1 and 2 dpi of TMV (B). The positions of genomic RNA (gRNA) and subgenomic RNA for CP (sgRNA) are indicated. Ethidium bromide-stained ribosomal RNA is presented as a loading control. The results from two independent experiments are shown. The accumulation of TMV RNA was also analyzed by real-time RT-PCR analysis using TMV CP-specific primers at 2 dpi of TMV (C). The accumulation level of ubiquitin mRNA was used as a reference. The mean level of TMV RNA in non-silenced leaves was taken as a standard (100%). (D–F) Fluorescent foci of TMV-GFP in non-silenced and *TARF*-silenced plants. TMV-GFP was inoculated to non-silenced and *TARF*-silenced plants and photographs were taken under UV irradiation at 7 dpi (D). Scale bars are 10 mm. The numbers (E) and area (F) of fluorescent foci of TMV-GFP in the inoculated leaves were analyzed using ImageJ software with images of 96 (in non-silenced leaves) and 490 (in *TARF*-silenced leaves) TMV-GFP fluorescent foci from eight leaves of four independent plants. The mean numbers and area with standard deviation are indicated, respectively. The asterisk indicates that the number of fluorescent foci was significantly different in non-silenced and TARF-silenced leaves ($P < 0.01$). Experiments were repeated at least three times with similar results.

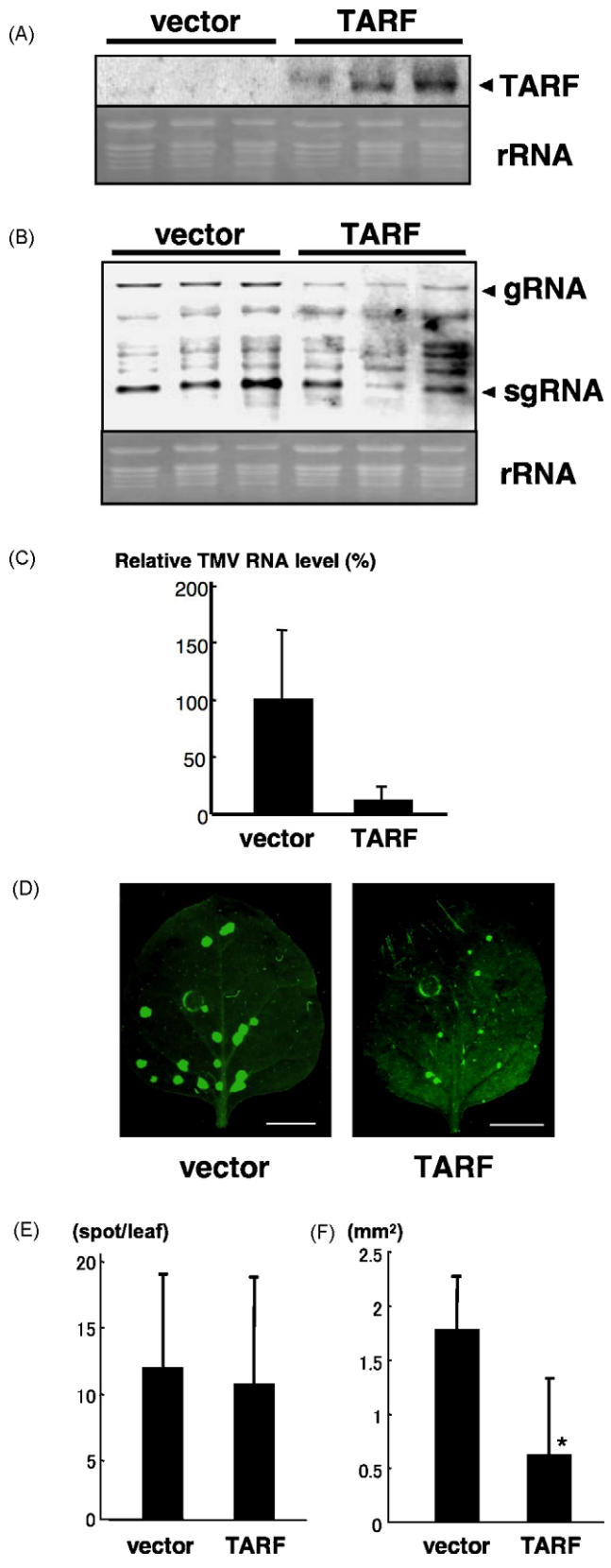

Fig. 4. TMV accumulation in *TARF*-overexpressed leaves. (A) *TARF* mRNA accumulation in *TARF*-agroinfiltrated leaves. Total RNA from pBI121 (vector) and pBI-TARF (TARF) agroinfiltrated *Nicotiana benthamiana* leaves at 2 days post-infiltration were analyzed by Northern blot analysis using TARF-specific probe. Ethidium bromide-stained ribosomal RNA is presented as a loading control. The results from three independent experiments are shown. (B and C) The accumulation of TMV RNA in *TARF*-agroinfiltrated leaves. Leaves agroinfiltrated with pBI121 or pBI-TARF at 2 days post-infiltration were inoculated by purified TMV particles. Total RNA from those leaves was analyzed by Northern blot analysis using TMV CP-specific probe (B). Ethidium bromide-stained ribosomal RNA is presented as a loading control. The results from three independent experiments are shown. The accumulation of TMV RNA was also analyzed by real-time RT-PCR analysis using TMV CP-specific primers at 2 dpi of TMV (C). The accumulation level of ubiquitin mRNA was used as a reference. The mean level of TMV RNA in vector-infiltrated leaves was taken as a standard (100%). (D–F) Fluorescent foci of TMV-GFP in agroinfiltrated leaves. TMV-GFP was inoculated to empty vector and pBI-TARF agroinfiltrated leaves at 2 days post-infiltration and photographs were taken under UV irradiation at 5 dpi (D). Scale bars are 10 mm. The numbers (E) and area (F) of fluorescent foci of TMV-GFP in the inoculated leaves were analyzed using ImageJ software with images of 137 (in vector agroinfiltrated leaves) and 121 (in pBI-TARF agroinfiltrated leaves) foci from twelve leaves of four plants in each experiment. The mean numbers and area with standard deviation are indicated, respectively. The asterisk indicates that the area of fluorescent foci was significantly different in vector and pBI-TARF agroinfiltrted leaves ($P < 0.01$).

1525–2109; TARF-CE) regions of TARF as well as C-terminus (nt 3204–3702; TARF-CT) (SFig. 1A), then TMV was secondarily inoculated to the silenced leaves. Northern blotting showed that the accumulation of TMV RNA was apparently higher in TARF-NT, TARF-CE and TARF-CT silenced leaves than in non-silenced leaves (SFig. 1B). When TMV-GFP was inoculated to silenced or non-silenced leaves, increased level of fluorescence by TMV-GFP was observed in each *TARF*-silenced leaves compared to that in non-silenced leaves (SFig. 1C). There was no difference in the effect of *TARF* silencing on TMV accumulation depending on the sites of silencing target in *TARF* ORF. These indicate that the silencing seems to target specifically to *TARF* and not to other closely related genes.

Next, we transiently expressed TARF in *N. benthamiana* and investigated the effect on TMV infection. The full-length cDNA of *TARF* was cloned into a binary vector, pBI121, and transiently expressed in *N. benthamiana* via agroinfiltration. TMV particles were inoculated to pBI-TARF or pBI121, an empty vector, delivered leaves. A transient high level of *TARF* expression was detected by Northern blotting in pBI-TARF agroinfiltrated leaves while undetectable level of expression in empty vector agroinfiltrated leaves (Fig. 4A). Northern blotting of TMV RNA showed that TMV accumulation was lower in the pBI-TARF agroinfiltrated leaves than in the empty vector agroinfiltrated leaves (Fig. 4B). Real-time RT-PCR analysis was performed to quantitatively analyze the accumulation of TMV RNA, and showed that the accumulation of TMV RNA in pBI-TARF agroinfiltrated leaves was approximately 12% of that in empty vector agroinfiltrated leaves (Fig. 4C). Furthermore, we inoculated TMV-GFP on the agroinfiltrated leaves to visualize the formation and spread of TMV-GFP foci. Fig. 4D shows the representative images of TMV-GFP foci, indicating that the level of GFP fluorescence in pBI-TARF agroinfiltrated leaves were lower than that in vector agroinfiltrated leaves. Interestingly, the difference of the size of TMV-GFP foci was much more obvious than that of the number of foci (Fig. 4E and F). These results indicated that TARF has a role that negatively regulates the accumulation of TMV.

3.3. Induction of TARF transcription by TMV infection

We investigated whether *TARF* transcription is induced by the infection of TMV. TMV particles were inoculated into *N. tabacum* cv Xanthi and the TMV-inoculated leaves together with mock-inoculated leaves were sequentially sampled for the detection of *TARF* transcript. Northern blotting analysis showed that *TARF* transcript was specifically induced in TMV-inoculated leaves (Fig. 5A). *TARF* transcript appeared to accumulate as rapidly as 4 h post-TMV inoculation. Real-time RT-PCR analysis confirmed the result by Northern blotting analysis (Fig. 5B). The accumulation of *TARF* transcript increased 4 h post-inoculation and decreased gradually. Thus, *TARF* expression was rapidly induced by the infection of TMV.

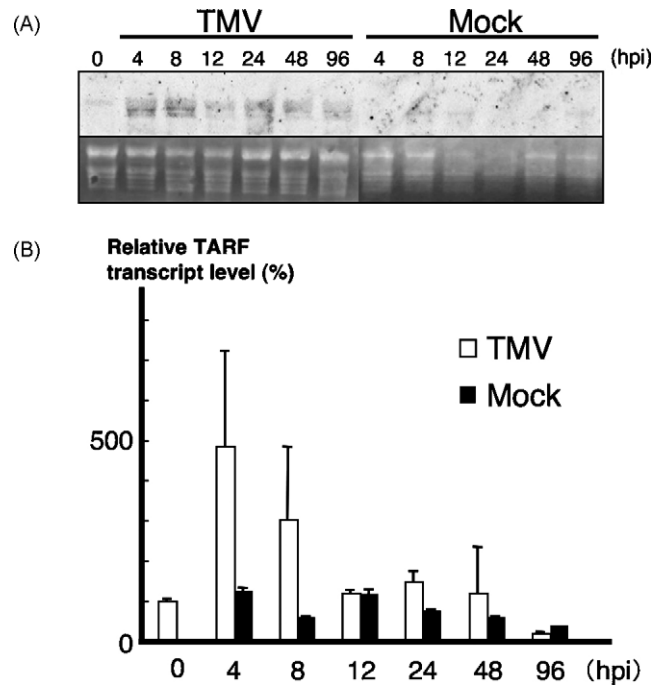

Fig. 5. The induction of TARF transcript. Total RNA from purified TMV particles (TMV) and mock-inoculated tobacco leaves were analyzed by Northern blot analysis using TARF-specific probe at indicated hours post-inoculation (A). Ethidium bromide-stained ribosomal RNA is presented as a loading control. The accumulation of TARF transcript was also analyzed by real-time RT-PCR analysis using TARF-specific primers (B). The accumulation level of ubiquitin mRNA was used as a reference. The mean level of TARF transcript in non-infiltrated leaves (0 hpi) was taken as a standard (100%).

4. Discussion

In this study, we presented a plant RING-H2 finger protein, TARF, that negatively regulates the accumulation of TMV. There have been reports of ubiquitin pathway components that disrupt the *R* gene-mediated HR triggered by a viral elicitor (González-Lamothe et al., 2006; Van den Burg et al., 2008) and reports of yeast ubiquitin pathway proteins that affect viral RNA accumulation (Li et al., 2008; Barajas et al., 2009). However, to our knowledge this report is the first presentation of a plant ubiquitin pathway component that actually regulates the accumulation of plant viral RNA.

TARF was a negative regulator of viral infection, but, surprisingly, LjnsRING, a *L. japonicus* orthologous gene of *TARF*, was essential for the symbiotic interaction between *M. loti* and *L. japonicus* (Shimomura et al., 2006). It was similar that TARF and LjnsRING were induced by the infection of TMV and *M. loti*, respectively. However, TARF has a negative effect on viral infection while LjnsRING positively regulates the infection of *M. loti*. In fact, the silencing of LjnsRING severely inhibited the nodule formation and the infection of *M. loti*. These may indicate that plants utilize the same ubiquitin pathway component during the interaction with microbes in a different manner depending on the pathogenic interaction or symbiotic interaction.

Present study indicated that TARF has an inhibitory effect on TMV infection in a TMV-susceptible host, *N. benthamiana* and that TARF transcript level was elevated by the inoculation of TMV. Tobacco ubiquitin-activating enzymes NtE1A and NtE1B are also induced by the inoculation of TMV (Takizawa et al., 2005). Therefore, the activation of the ubiquitin pathway may have an essential role in the resistance to viruses. Since these results are obtained in the compatible interaction between the plant and virus, the resistance to viruses dependent on the ubiquitin pathway may be one of the basal defense responses (Bent and Mackey, 2007). However, it is also possible that it has a role in the incompatible interaction because some E3s are involved in the *N* gene-mediated resistance induced by P50 (González-Lamothe et al., 2006; Van den Burg et al., 2008). It is interesting to study whether TARF is involved in the *N* gene-mediated resistance to TMV.

Although a number of ubiquitin ligases have been isolated as components of the defense pathway against pathogens and some of them had actually ubiquitin ligase activity (Takai et al., 2002; Zeng et al., 2004; Kawasaki et al., 2005; Yang et al., 2006; Hondo et al., 2007; Trujillo et al., 2008), the targets of most of them for the ubiquitination and/or proteolysis by the proteasome have not been revealed so far. Since the RING-H2 motif of TARF is quite similar to that of EL5 which has *in vitro* ubiquitin ligase activity (Takai et al., 2002), it is likely that TARF has the activity. In this study we revealed that TARF interacts with the RdRp of TMV, therefore TARF may recognize and ubiquitinate the RdRp of TMV. Consistent with this hypothesis, immuno-electron microscopy analysis showed that ubiquitin and/or ubiquitin conjugates colocalized with the virus replication complex, where viral RdRp was extensively detected in TMV infected cells (Gaspar et al., 1990). Similarly, the RdRp, in particular the P domain, of TYMV is polyubiquitinated in the heterologous expression system (Héricourt et al., 2000). Since TMV MP and CP have been indicated to be ubiquitinated in plant tissues, it would be interesting to determine whether TARF is involved in the ubiquitination of them.

The most attractive idea of the role of TARF in the resistance to TMV is that the RdRp of TMV is degradated by the proteasome through the ubiquitination by TARF. This hypothesis is consistent with the role of the yeast HECT-type E3, Rsp5p, that ubiquitinates and degrades the RdRp of TBSV (Barajas et al., 2009). The RdRp of plant RNA viruses are translated immediately after the virion disassembly and is required for genomic RNA synthesis earlier than the translation of other viral proteins (Buck, 1996). Therefore, it is expected that the strategy to degrade the viral RdRp is quite efficient, because it can inhibit virus accumulation at a very early stage of viral infection. In fact, *TARF* was rapidly induced by the inoculation of TMV. Moreover, the silencing of *TARF* increased the number of TMV-GFP foci indicating that TARF functions at a very early stage when it is determined whether viruses can establish a successful infection in the primary invaded cells or not. However, when TARF was transiently expressed, the size rather than the number of TMV-GFP foci was extensively suppressed. This may imply that only the expression of TARF has a certain negative effect on TMV-GFP infection, but is not sufficient to exhibit a rapid or high level of resistance to completely inhibit the formation of TMV-GFP foci, resulting in the appearance of small TMV-GFP foci. One possible explanation is that since tobacco E1 genes, *NtE1A* and *NtE1B*, are induced by the inoculation of TMV (Takizawa et al., 2005), a coordinated activation of E1, E2 and TARF may be essential for the exhibition of a high level of resistance.

The other hypothesis is that the ubiquitinated form of the RdRp is not degraded but recognized by the plant defense pathway. The modified form of plant virus elicitors is required for the recognition by the host resistance (Kim and Palukaitis, 1997; Hajimorad et al., 2005). It is also possible that TARF is a regulator in a defense pathway that targets and disrupts TMV infection and TMV RdRp may have a role to suppress the activity of TARF via its interaction with TARF. In this case, the target of TARF for ubiquitination and degradation is not the RdRp of TMV but may be a negative regulator in the resistance pathway to TMV.

To verify these hypotheses and reveal the role of TARF, it is necessary to analyze whether TARF has a ubiquitin ligase activity and targets and degrades the RdRp of TMV in the future research. It is also necessary to reveal the upstream pathway of TARF including E1 and E2 to elucidate how they can recognize the infection of TMV in the early stage of its infection. These findings together with

our present results will help to clarify a novel mechanism of virus resistance pathway in plants and to reveal other ubiquitin pathway components that are involved in virus resistance.

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