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Molecules and Cells

Arabidopsis TTR1 Causes LRR-Dependent Lethal Systemic Necrosis, rather than Systemic Acquired Resistance, to Tobacco Ringspot Virus

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Most *Arabidopsis* ecotypes display tolerance to the *Tobacco ringspot virus* (TRSV), but a subset of *Arabidopsis* ecotypes, including Estland (Est), develop lethal systemic necrosis (LSN), which differs from the localized hypersensitive responses (HRs) or systemic acquired resistance (SAR) characteristic of incompatible reactions. Neither viral replication nor the systemic movement of TRSV was restricted in tolerant or sensitive *Arabidopsis* ecotypes; therefore, the LSN phenotype shown in the sensitive ecotypes might not be due to viral accumulation. In the present study, we identified the Est *TTR1* gene (tolerance to *Tobacco ringspot virus 1*) encoding a TIR-NBS-LRR protein that controls the ecotype-dependent tolerant/sensitive phenotypes by a map-based cloning method. The tolerant Col-0 ecotype *Arabidopsis* transformed with the sensitive Est *TTR1* allele developed an LSN phenotype upon TRSV infection, suggesting that the Est *TTR1* allele is dominant over the tolerant *ttr1* allele of Col-0. Multiple sequence alignments of 10 tolerant ecotypes from those of eight sensitive ecotypes showed that 10 LRR amino acid polymorphisms were consistently distributed across the *TTR1*/*ttr1* alleles. Site-directed mutagenesis of these amino acids in the LRR region revealed that two sites, L956S and K1124Q, completely abolished the LSN phenotype. VIGS study revealed that TTR1 is dependent on SGT1, rather than EDS1. The LSN phenotype by TTR1 was shown to be transferred to *Nicotiana benthamiana*, demonstrating functional conservation of TTR1 across plant families, which are involved in SGT-dependent defense responses, rather than EDS1-dependent signaling pathways.

INTRODUCTION

Plants have evolved an array of defense mechanisms to counter challenges from a wide range of pathogens. As an initial defense, plants express proteins that recognize and induce nonspecific resistance responses to pathogen-associated molecular patterns (PAMP), similar to the innate immune response of animals (Jones and Dangl, 2006). Next, plants express *resistance* (*R*) genes that directly or indirectly recognize the products of pathogen-specific *avirulence* (*Avr*) genes to mediate gene-for-gene resistance (Flor, 1971). *R* gene-mediated pathogen recognition triggers downstream defense responses, which commonly include "rapid" apoptotic death of infected plant cells, manifesting as a hypersensitive response (HR) to minimize the spread of disease local (Hulbert et al., 2001), and ultimately leads to whole plant resistance response, systemic acquired resistance (SAR). SAR is associated with the induction of a *pathogen-related (PR)* genes and accumulation of endogenous salicylic acid (SA). Systemic necrosis, on the other hand, often found as sensitive responses in both plants and animal systems to pathogens, instead of HR. A phenomenon of lethal systemic necrosis (LSN) that *R* gene induces systemic cell deaths even with the induction of PR gene expression has not been reported yet.

Most characterized plant *R* genes show similar structures of nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Bent and Mackey, 2007; Jones and Dangl, 2006). The *Arabidopsis thaliana* genome contains approximately 149 NBS-LRR-encoding genes (Meyers et al., 2003). Some plant species with larger genomes (e.g., *Medicago truncatula*, *Oryza sativa*, and *Populus trichocarpa*) have more than 400 NBS-LRR encoding sequences in their genomes (Kohler et al., 2008; Zhou et al., 2004). NBS-LRR type *R* genes can be grouped into two classes: the CC-NBS-LRR (CNL) type with a coiled-coil (CC) domain and the TIR-NBS-LRR (TNL) type with an orthologous N-terminal domain consisting of *Drosophila Toll* and mammalian *Interleukin-1 receptor* (TIR). Fifty-five "CNL" type *R* genes and 94 "TNL" type *R* genes in *Arabidopsis* have been

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reported (Hulbert et al., 2001), and each type of R protein interacts with different downstream signaling components (NDR1 and EDS1, respectively) to trigger defense responses (Aarts et al., 1998). NBS-LRR domains of *RPS5* gene in *Arabidopsis* are thought to play a role of recognizing and interacting with *AvrPphB*, a bacterial protease (Ade et al., 2007). AVR-Pita protein binds specifically to the Leucine-rich domain (LRD) at the C-terminus of the Pi-ta protein, which approximately corresponds to the LRR domain of the RPM1 protein, both in the yeast two-hybrid system and in an *in vitro* binding assay (Jia et al., 2000). The full-length RRS1-R, a TIR-NBS-LRR protein conferring resistance to bacterial wilt, interacts with PopP2, a type III effector of *R. solanacearum* GMI1000, and NBS-LRR domain may intercepts the PopP2 effector to fulfill the function as a cytoplasmic guard protein (Deslandes et al., 2003). The tobacco *N* gene, well-known TIR-NBS-LRR class of *R* gene, confers resistance to TMV (Dinesh-Kumar et al., 1995). Some loss-of-function *N* alleles such as the TIR deletion and point mutations in the NBS region were found to induce systemic hypersensitive response (SHR), similar phenotype as LSN, by allowing TMV to move systemically within tobacco plants (Dinesh-Kumar et al., 2000). Recently, Stange et al. (2008) reported that five non-conservative substitutions in the LRR regions of Tobacco *N* gene and *N homolog (NH)* gene result in differential ligand recognition properties.

Although NBS-LRR proteins are mostly associated with disease resistance, they have also been linked to disease sensitivity in plants as well as hypersensitive human genetic disorders. In humans, a family of proteins containing nucleotide-binding oligomerization domains (NODs) is involved in disease responses and apoptosis (Inohara et al., 1999). Mutations in the NBS region of Nod2 induce the constitutive activation of NF-κB, which is responsible for Blau syndrome, an auto-inflammatory disease (Chamaillard et al., 2003). However, when *Nod2* is over-stimulated, it is associated with the chronic intestinal inflammatory disorder Crohn's disease (Wilmanski et al., 2008; Ye and Ting, 2008). These studies suggest that NBS-LRR proteins can initiate signaling cascades that result in disease, rather than resistance. The innate immune system is the most ancestral response against a variety of microbial challenges and acts as a basal defense system in both plants and animals (Bent and Mackey, 2007; Jones and Dangl, 2006; Medzhitov, 2007).

Tobacco ringspot virus (TRSV) is a member of the *Nepovirus* genus in the family Comoviridae, and it infects a wide range of dicotyledonous plants (Chu and Francki, 1979). Of 97 *Arabidopsis* ecotypes tested, most are tolerant and show no obvious disease symptoms to TRSV infection, but a subset of ecotypes are sensitive and develop lethal systemic necrosis (LSN), leading rapidly to death (Lee et al., 1996). A single gene, *tolerance to tobacco ringspot virus 1* (*TTR1*), is thought to be responsible for both the common tolerant phenotype and the necrotic responses seen in the sensitive phenotype (Lee et al., 1996). In this study, we isolated *TTR1*, which encodes a "TNL"-type protein. Molecular mechanisms of *R* gene-mediated tolerance and/or sensitivity in the *Arabidopsis*-TRSV interaction were studied and we show evidence that *TTR1*-elicited responses are the result of impaired *R* gene-mediated disease responses.

MATERIALS AND METHODS

Plant materials and TRSV inoculations

Arabidopsis thaliana and *Nicotiana benthamiana* plants were grown in a growth chamber (23°C, 16 h light/8 h dark) for 3 weeks prior to TRSV inoculation. Disease phenotypes against TRSV were evaluated at 5-15 dpi (days post inoculation) and 5-7 dpi, respectively. TRSV cultures were maintained and inoculations performed as previously described (Lee et al., 1996; Steere, 1956). The *Arabidopsis* ecotypes Aa-0, Bsch-0, Bur-0, Cal-0, Co-0, Col-0, Col-4, Cvi-0, Est, Ha-0, H55, Ka-0, Kn-0, Lc-0, Ms-0, Nd-0, Np-0, and Ws-0 lines were obtained from the Arabidopsis Biological Resource Center (ABRC).

Genetic analysis

Reciprocal crosses were made between Est and Col-0 *Arabidopsis* ecotypes. F_1 and F_2 progenies of each cross were examined for the disease phenotypes following TRSV inoculation. The segregation ratios of disease phenotypes from each cross were recorded and confirmed by PCR using SSLP markers, which were designed within the region flanking the *TTR1* sequence based on information from the Arabidopsis Information Resource (TAIR).

PCR amplification and map-based cloning

Polymerase chain reaction (PCR) was performed using a PTC-200 cycler (MJ Research, USA) with the following conditions: one cycle at 95°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finishing at 72°C for 5 min. *TTR1* was mapped between the nga129 and DFR markers on chromosome V, and co-segregated with the yUP5F5LE marker on the yUP5F5 YAC clone (Lee et al., 1996). Approximately 1,500 Col-0 × Est F_2 plants were screened for recombinants between the X15S and SMS markers. Subsequently, the markers Y68C and Z67S between X15S and SMS were used to identify polymorphisms between Col-0 and Est. CAPS and SSLP markers used in this study are listed in the Supplementary Table 1. The genotypes of F_2 individuals at the *TTR1* locus were determined by TRSV inoculation.

Construction of fosmid and subclone libraries

Genomic DNA was isolated from the 3-week-old Est *Arabidopsis* ecotype as described (Sambrook et al., 2001) and partially digested with *Sau*3AI for size fractionation (Takara, Japan). End-repaired dephosphorylated 30-40-kb DNA fragments were ligated into the *Bam*HI site of the fosmid vector pCC1FO (Epicentre Biotechnologies, USA). Packaging into phages and transfection into *Escherichia coli* EPI300-T1 were performed according to the manufacturer's protocol. Fosmid library were screened and four fosmid clones (418-17, 942-19, 2411-19, 1506-2) were selected. The digested fragments of these fosmid clones were subcloned into a pPZP211 binary vector for plant transformation (Hajdukiewicz et al., 1994).

The nucleotide sequence of *Arabidopsis thaliana* ecotype Estland (Est) *TTR1* has GenBank accession number FJ613384. The nucleotide sequence of various *Arabidopsis* ecotypes *TTR1* partial mRNA described in this study has GenBank accession numbers FJ613385-FJ613402.

Agrobacterium-mediated plant transformation

Arabidopsis Col-0 ecotype plants were transformed with 27 genomic subclones of Est ecotypes using an *Agrobacterium tumefaciens*-mediated transformation method (Clough and Bent, 1998). *N. benthamiana* plants were also transformed with a clone (7-21) by *A. tumefaciens*-mediated transformation (Gallois and Marinho, 1995).

Northern blot analysis

Three-week-old Col-0 and Est *Arabidopsis* were inoculated with TRSV, and two leaves per ecotype were harvested from 0 to 7 dpi. Total RNA was extracted from these leaf tissues using TRI

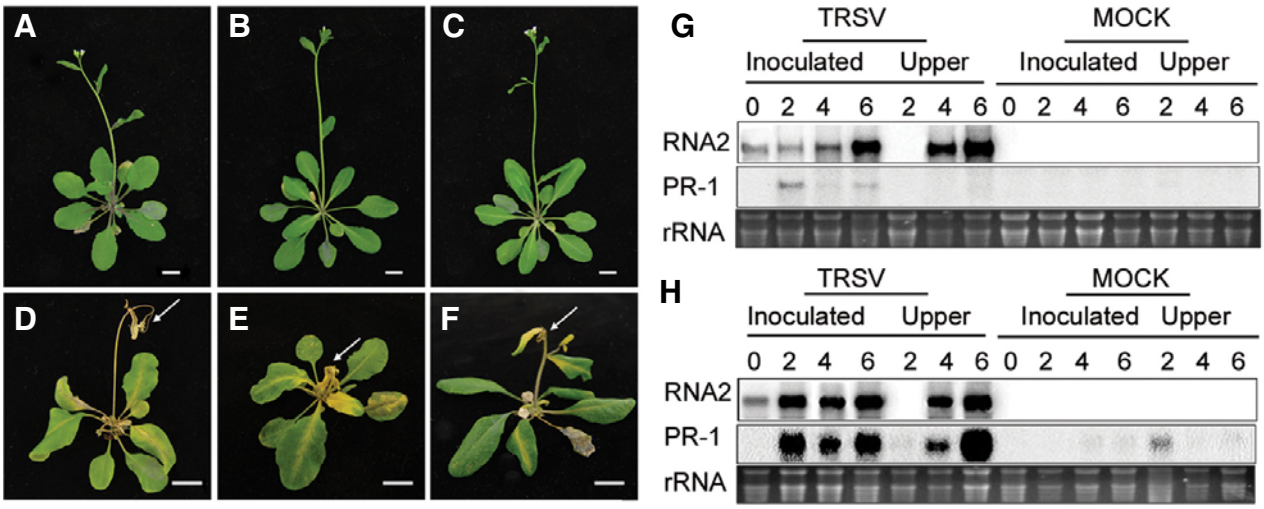

Fig. 1. *Arabidopsis* ecotype-specific tolerant/sensitive responses upon TRSV inoculations. The tolerant Col-0 (A), Aa-0 (B), Ka-0 (C), and sensitive Est (D), Bsch-0 (E), and H55 (F) exhibited distinct disease phenotypes. Lethal systemic necrosis (LSN) phenotype are shown in all inoculated leaves (arrows), systemic upper leaves (*), and shoot apical meristems of the sensitive ecotypes. Northern blot analysis reveals TRSV RNA2 and *Arabidopsis PR1* gene expression after TRSV inoculation in tolerant Col-0 (G) and sensitive Est ecotypes (H). Scale bars = 1 cm.

reagent (Molecular Research Center, Inc., USA), according to the manufacturer's protocol. TRSV viral CP encoding DNA and *PR1*-encoding DNA were prepared by PCR and labeled with ^{32}[P]-dCTP using the Multiprime DNA Labeling System (Amersham Pharmacia, USA). Northern blotting and hybridization were performed as described (Sambrook et al., 2001).

Sequence analyses

Amino acid sequences of the tolerant and sensitive *Arabidopsis* ecotypes were analyzed using the DNAMAN software (Lynnon Co., Canada). The LRR regions of the *Arabidopsis* ecotypes were PCR-amplified by 3′-RACE (Gibco-BRL, USA). The gene-specific primers used to amplify the LRR regions of various ecotypes are shown in Supplementary Table 1.

Site-directed mutagenesis

Single amino acid substitutions in the LRR regions of TTR1 were generated using the QuickChange kit (Stratagene, USA) according to the manufacturer's protocol. A *Spe*I/*Sal*I fragment of about 2 kb of the LRR region of *TTR1* was subcloned into a T vector (RBC Co., Taiwan) to create a master clone (~5 kb). PCR was performed to introduce a point mutation at each desired site in the LRR region using two synthetic oligonucleotide primers (Supplementary Table 1). Single amino acid substitutions of all 10 *TTR1*^{M} clones were confirmed by direct DNA sequencing.

Virus-induced gene silencing (VIGS)

Three-week-old *N. benthamiana* plants expressing *TTR1* were agroinfiltrated with TRV derivatives as previously described (Ratcliff et al., 2001). *Agrobacterium* with TRV2:pPZP211 vector, TRV2:PDS, and TRV2:GFP constructs were mixed with TRV at a 1:1 ratio and infiltrated individually on the cotyledons of transgenic *N. benthamiana* as experimental controls (Chung et al., 2004). Nb*SGT1*, Nb*HSP90*, Nb*RAR1*, Nb*EDS1*, and Nb*PAL* were individually silenced by VIGS for 7 days and TRSV was challenged on the upper leaves of the plants with specific genes silenced for evaluation of the disease phenotypes at 5 dpi. Total RNA was isolated from the *N. benthamiana* leaf and each target mRNA expression level was determined by RT-PCR. The *N. benthamiana actin* mRNA was used as a positive control.

RESULTS

TRSV disease symptoms in *Arabidopsis* ecotypes

Three-week-old *Arabidopsis* ecotypes Aa-0, Bsch-0, Col-0, H55, Est, and Ka-0 were inoculated with TRSV. Aa-0, Col-0, and Ka-0 exhibited tolerance toward TRSV infection displaying no obvious disease symptoms on the inoculated or systemic upper leaves (Figs. 1A-1C). However, ecotypes Bsch-0, Est, and H55 showed necrotic symptoms 4 dpi. Within 2 weeks of TRSV inoculation, shoot apical meristems and flower buds of these sensitive ecotypes completely withered, and growing tips of plants were characteristically hooked (Figs. 1D-1F), indicating that lethal systemic necrosis (LSN) occurred. The LSN response shown in ecotypes Bsch-0, Est, and H55, was different from the local lesions often associated with a localized HR in resistant plants, or from plants expressing systemic acquired resistance (SAR).

Reciprocal crosses made between the tolerant ecotype Col-0 and the sensitive ecotype Est showed that all of the F_1 progeny developed LSN following inoculation with TRSV. In F_2 populations, tolerance and sensitivity to TRSV infection segregated in a 1:3 ratio (tolerant:sensitive; Supplementary Table 2). Thus we conclude that the allele for sensitivity to TRSV infection (*TTR1*) from Est was dominant over the allele for tolerance to TRSV infection (*ttr1*) from Col-0.

To determine whether the LSN responses of the sensitive *Arabidopsis* ecotypes were associated with the movement of TRSV, the movement of the virus was monitored over time in TRSV-inoculated Col-0 (Fig. 1G) and Est (Fig. 1H) plants by northern blot analysis using a probe corresponding to the region of RNA 2 (encoding the coat protein). In TRSV-inoculated plants, TRSV RNA was detected by 4 dpi in systemic upper leaves, and the viral RNA accumulation increased to compara-

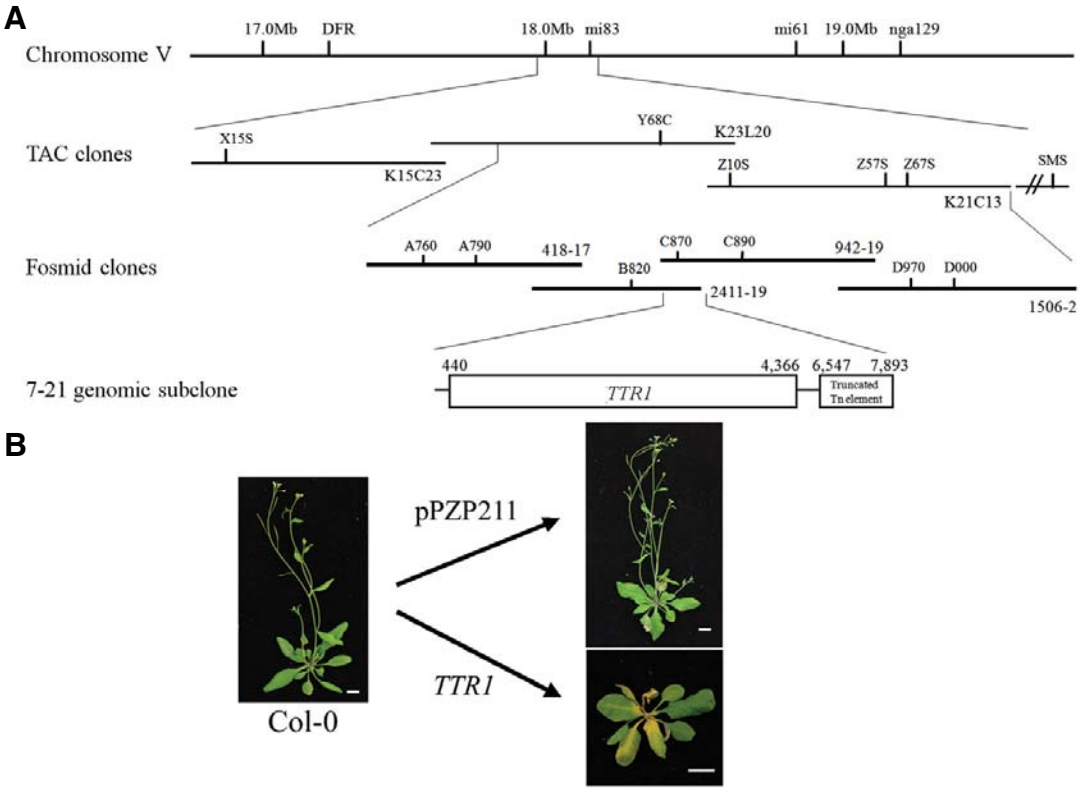

Fig. 2. Map-based cloning of *TTR1* of *Arabidopsis* ecotype Est. (A) Schematic representations of the map-based cloning of *TTR1*. Contigs of three TAC clones and four fosmid clones were first identified and aligned near the DFR and nga129 markers. Subclones ($n = 27$) were generated from these fosmid clones, and each subclone was transformed into Col-0. Plants of each transgenic line were evaluated for the disease phenotype following TRSV inoculation. (B) Subclone 7-21, containing a single ORF of Est ecotype (*TTR1*) and a truncated transposable element, made the normally tolerant Col-0 sensitive to TRSV, whereas Col-0 transformed with the pPZP211 binary vector retained its tolerant phenotype. A single ORF *TTR1* was reintroduced into the Col-0 background, and the LSN phenotype was confirmed in T_1 plants.

ble levels in inoculated and systemic upper leaves of both tolerant Col-0 and sensitive Est until 6 dpi, when the TRSV-infected Est plants became necrotic (Figs. 1G and 1H). The mRNA encoding *pathogenesis-related protein 1 (PR-1)* accumulated to much higher levels in the sensitive ecotype Est than in the tolerant ecotype Col-0, in both inoculated and systemic leaves (Figs. 1G and 1H). At 6 dpi, *PR-1* expression was even greater in the systemic upper leaves than in inoculated leaves (Fig. 1H). Thus, the LSN phenotype was associated with the induction of plant defense pathways.

TTR1 encodes a TIR-NBS-LRR protein

A map-based cloning approach was used to identify the gene that confers sensitivity (LSN) to TRSV infection in Est ecotype *Arabidopsis*. *TTR1* was previously mapped to chromosome V, between nga129 and *DFR* (Lee et al., 1996). For this genetic interval, cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers (Supplementary Table 1) were designed between X15S and SMS, and used to fine-map *TTR1* in a population of 1,500 F_2 plants from crosses between Col-0 and Est. From this population, 30 F_2-derived lines were identified that contained recombinations between *TTR1*/SSLP and CAPS markers, and *TTR1* was mapped between the markers Y68C and Z67S in the TAC clones (K23L20 and K21C13). A fosmid library of Est genomic DNA was constructed, and four fosmid clones (418-17, 942-19, 1506-2, and 2411-19) proximal to the TAC clones (K23L20 and K21C13) were selected by PCR using CAPS and SSLP markers (Supplementary Table 1). These fosmid clones were subcloned into the pPZP211 vector (Hajdukiewicz et al., 1994), and Col-0 plants transformed with one subclone, 7-21, responded to TRSV inoculation with the LSN phenotype, indicating that the clone contains the gene representing *TTR1* (Fig. 2A). Sequence analysis of the 7-21 subclone showed high sequence identity to a complete open reading frame of Col-0, At5g44870 (1,170 amino acids), with five exons, four introns, and a truncated transposable element. The ORF of the 7-21 clone (*TTR1*) was re-transformed into Col-0, and LSN phenotype was reproduced upon TRSV infection (Fig. 2B), concluding that this ORF (Est *TTR1*) is responsible for LSN. Est *TTR1* encodes a putative protein of 1,163 amino acids and resembles the "TNL"-type *R* genes (Fig. 3).

Tolerance/sensitivity determined by the TTR1 LRR amino acid sequence

The predicted amino acid sequences of the TIR and NBS regions of the Est and Col-0 TTR1 proteins were identical, but the LRR regions differed at 21 positions (Fig. 3), indicating that the LRR region are responsible for the sensitivity/tolerance responses of each ecotype to TRSV.

We further evaluated 18 *Arabidopsis* ecotypes, 10 sensitive (Bsch-0, Bur-0, Cal-0, Co-0, Cvi-0, Est, H55, Ha-0, Ms-0, Np-0) and 8 tolerant (Aa-0, Col-0, Col-4, Ka-0, Kn-0, Lc-0, Nd-0, Ws-0), for comparison of the predicted LRR regions. Partial amino

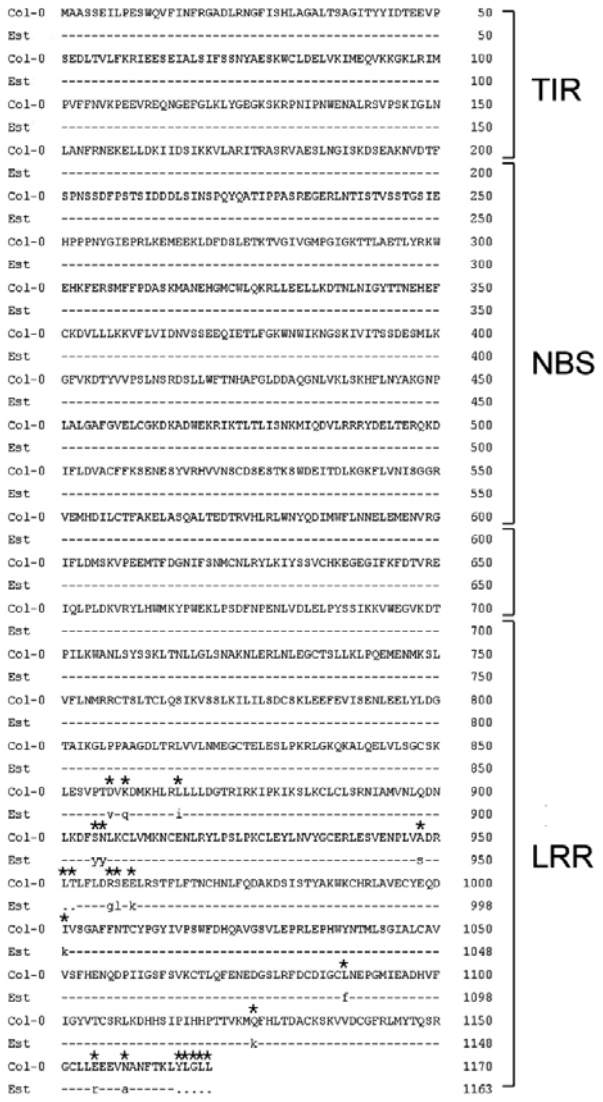

Fig. 3. Comparison of the *TTR1* (Est) and *ttr1* (Col-0) amino acid sequences. *TTR*1 encodes a "TNL"-type gene. The predicted sequences of TTR1 in Est and ttr1in Col-0 were aligned and compared using the DNAMAN software. The sequences of the TIR-NBS regions in Est and Col-0 were identical, but 21 amino acid differences were observed in the LRR region. Identical amino acids are marked by dashes (-), and amino acid differences are indicated by asterisks (*).

acid sequences of these 18 ecotypes were amplified by 3′-rapid amplification of cDNA ends (3′-RACE), and direct DNA sequencing of the LRR regions (amino acid positions 851-1,163) revealed 10 amino acid changes that were conserved in all sensitive ecotypes and absent from all tolerant ecotypes (Fig. 4A). These changes are considered as potentially responsible for the LSN response to TRSV infection. A site-directed mutagenesis approach was taken to replace the unique 10 amino acid residues of sensitive *TTR1* individually with those from tolerant *ttr1* (Figs. 4A and 4B). One or more of each mutated *TTR1*^{M} clone was introduced into tolerant Col-0 and the phenotype was evaluated after TRSV infection. As shown in Fig. 4C, Col-0 plants transformed with any one of the eight *TTR1*^{M} clones (V857D, I866L, Y905S, G955R, K958E, K999I, R1153E, A1157N) showed the LSN phenotype upon TRSV inoculation, suggesting that these eight residues of *ttr1* are not directly involved in LSN (Fig. 4C). However, Col-0 plants transformed with the L956S or K1124Q clones remained tolerant to TRSV (Fig. 4C), indicating that these two amino acid residues are essential for TTR1 function.

TTR1 is functional across plant families

A. thaliana is in the family Brassicaceae, and we decided to investigate *TTR1* function in *N. benthamiana*, which is in the family Solanaceae and is a model system for studying plant-virus interactions (Goodin et al., 2008). We found that wild-type *N. benthamiana* and *N. benthamiana* transformed with the pPZP211 binary vector were tolerant to TRSV (Fig. 5A), but *N. benthamiana* plants transformed with subclone 7-21 (Est *TTR1*) showed the LSN phenotype upon infection with TRSV (Fig. 5B), suggesting that TTR1 detrimentally interacted with downstream defense pathways in *N. benthamiana*, as seen in *A. thaliana.*

We further tested the roles of five genes (Nb*EDS1*, Nb*SGT1*, Nb*RAR1*, Nb*Hsp90*, and Nb*PAL*) in different defense-related signaling pathways for *TTR1*-mediated LSN *via* a *Tobacco rattle virus* (TRV) virus-induced silencing (VIGS) in *N. benthamiana* expressing *Arabidopsis* TTR1 with a pPZP211 vector and two foreign genes, *phytoene desaturase* (*PDS*) and green fluorescence protein (GFP) as VIGS control (Fig. 6). Plastid localized enzyme, *PDS* converts phytoene to colored ξ-carotene in carotenoid biosynthesis pathway (Wetzel and Rodermel, 1998). VIGS with pPZP211 vector (Fig. 6A) showed no phenotypical changes on growth of *N. benthamiana*, while silencing of *PDS* (Fig. 6B) exhibited whitening of leaves indicating successful VIGS of a target gene. Silencing of each defense related gene (Nb*SGT1*, Nb*RAR1*, Nb*EDS1*, and Nb*PAL1*) was also shown by significant reductions in the accumulation of the cognate mRNAs, implying successful silencing of the target (Fig. 7). Notably, no tolerant responses were observed in plants co-infected with TRSV and TRV2 containing GFP, Nb*EDS1*, Nb*Hsp90*, Nb*PAL*, or Nb*RAR1* (Figs. 6C, and 6E-6H). However, all plants co-inoculated with TRSV and TRV2 containing *SGT1* were tolerant to TRSV infection (Fig. 6D), indicating that only *SGT1* is involved in LSN. If this is true, the *Arabidopsis* TTR1 may operate to induce LSN *via* defense-related signaling pathways that exclusively involve *SGT1*, not *Hsp90/RAR1*. TTR1 mediated LSN is still remained to be elucidated, especially how to relay information leading to LSN with potential downstream components.

DISCUSSION

To understand the molecular mechanisms underlying the ecotype-specific tolerance/sensitivity to TRSV, the *TTR1* gene from the *Arabidopsis* ecotype Est, which induces LSN, was isolated by map-based cloning. We confirmed that the "TNL"-type Est *TTR1* (a counterpart of Col-0 At5g44870, *ttr1*) indeed induces LSN by over-expression of the ORF of Est *TTR1* with native promoter into the tolerant ecotype Col-0 in response to TRSV. Distinct amino acid changes were found between positions 823 and 1,170 of the LRR region of TTR1/ttr1, clearly dividing the *Arabidopsis* ecotypes into sensitive and tolerant subgroups (Fig. 2A). Amino acid sequence variations in the LRR regions of R proteins are believed to facilitate protein recognition of a variety of pathogen-derived molecules (Hulbert et al., 2001; Meyers et al., 2003).

In this study, we identified two amino acids in the LRR region that are responsible for sensitive ecotype-specific LSN re-

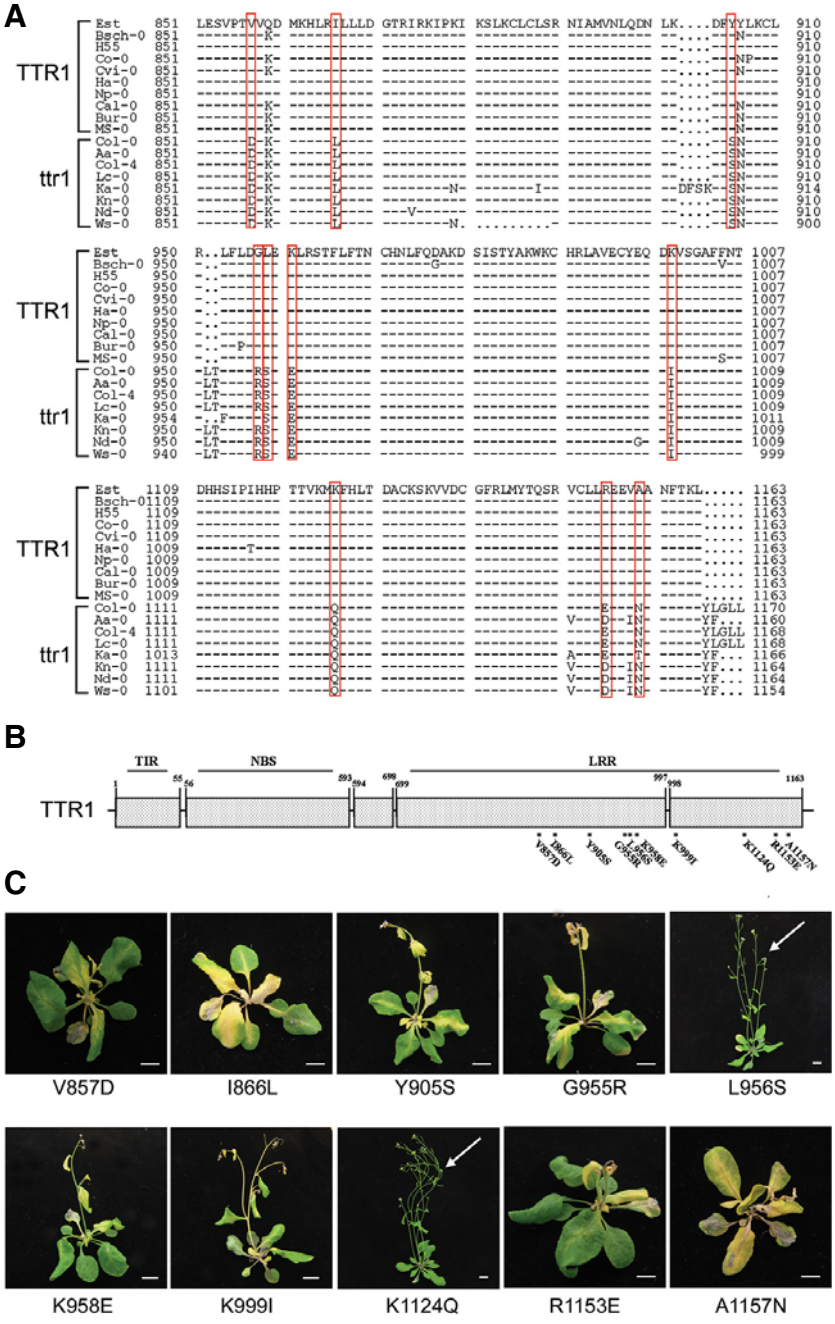

Fig. 4. The LRR region of TTR1/ttr1 determines ecotype-specific responses to TRSV. (A) Multiple sequence alignments of the LRR regions of *TTR1/ttr1* loci from multiple *Arabidopsis* ecotypes, separating *A. thaliana* ecotypes into two groups. (B) Schematic representation of *TTR1*, a "TNL"-type gene with five exons, and point mutations used to determine the role of TTR1 in causing LSN. (C) Each mutated clone (*TTR1*^{M}) was introduced into Col-0 by *Agrobacterium*-mediated transformation. Plants transformed with L956S and/or K1124Q showed tolerance to TRSV, indicating the loss of TTR1 function. Scale bars = 1 cm.

sponses to TRSV. Stange et al. (2008) reported that five non-conservative substitutions in the LRR regions of Tobacco N gene and *NH* gene (Gly81 → Arg80, Leu153 → Asp152, Arg198 → Glu197, Val99 → Glu198 and Ile221 → Lys220) result in differential ligand recognition properties. Comparative three dimensional models of N-LRR and NH-LRR proposed that the LRR region is important in pathogen recognition (Stange et al., 2008). Thus, we could postulate that two residues in the LRR region of TTR1, Leu956 and Lys1124, identified in this study as responsible amino acids for LSN phenotype of TTR1 might also be important for the proper LRR domain folding, leading to stable or transient recognition of TRSV-derived molecules.

SAR is an inducible broad spectrum plant defense response that requires the accumulation of SA. Many "TNL"-type R proteins require EDS1, not NDR1, for defense response activities (Lee et al., 1996; Peart et al., 2002), leading to HR or SAR. As TTR1 function was shown to be transferred to another plant family, the Solanaceae (*N. benthamiana*), we utilized VIGS to further characterize the relationship between TTR1 and five known signaling components downstream. Interestingly, the VIGS results showed that TTR1 was not regulated by Nb*EDS1*-dependent manner, but Nb*SGT1*-dependent, suggesting that TTR1 might utilize unique regulatory systems to respond TRSV, as opposed to use the SA mediated signaling pathway by most known *R* genes. It has been reported that

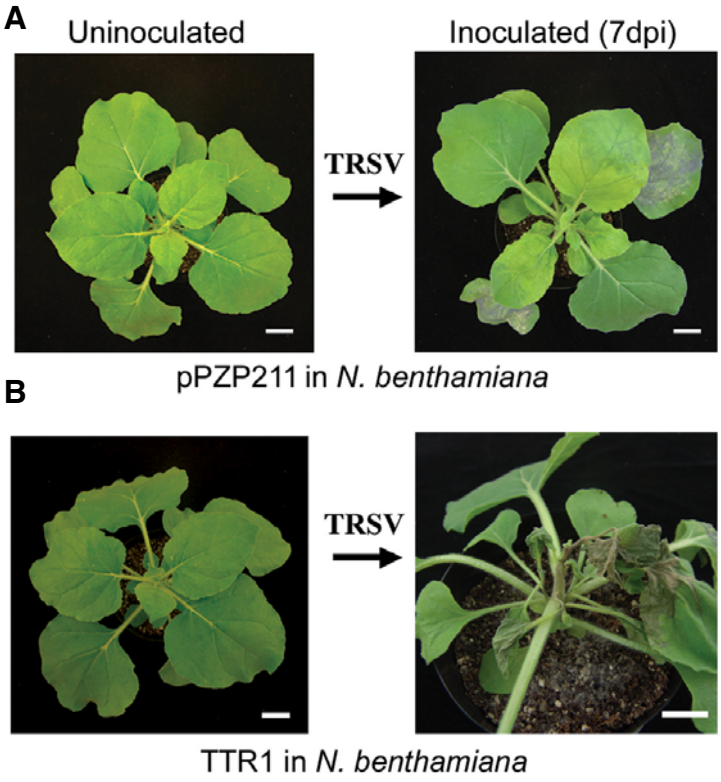

Fig. 5. *Arabidopsis* TTR1 causes LSN in *N. benthamiana* upon TRSV inoculation. (A) *N. benthamiana* transformed with the empty pPZP211 binary vector displays no obvious disease symptoms in response to TRSV. (B) *N. benthamiana* transformed with pPZP211 expressing Est *TTR1* exhibited LSN (7 dpi), as seen in sensitive *Arabidopsis* ecotypes. Scale bars = 2 cm.

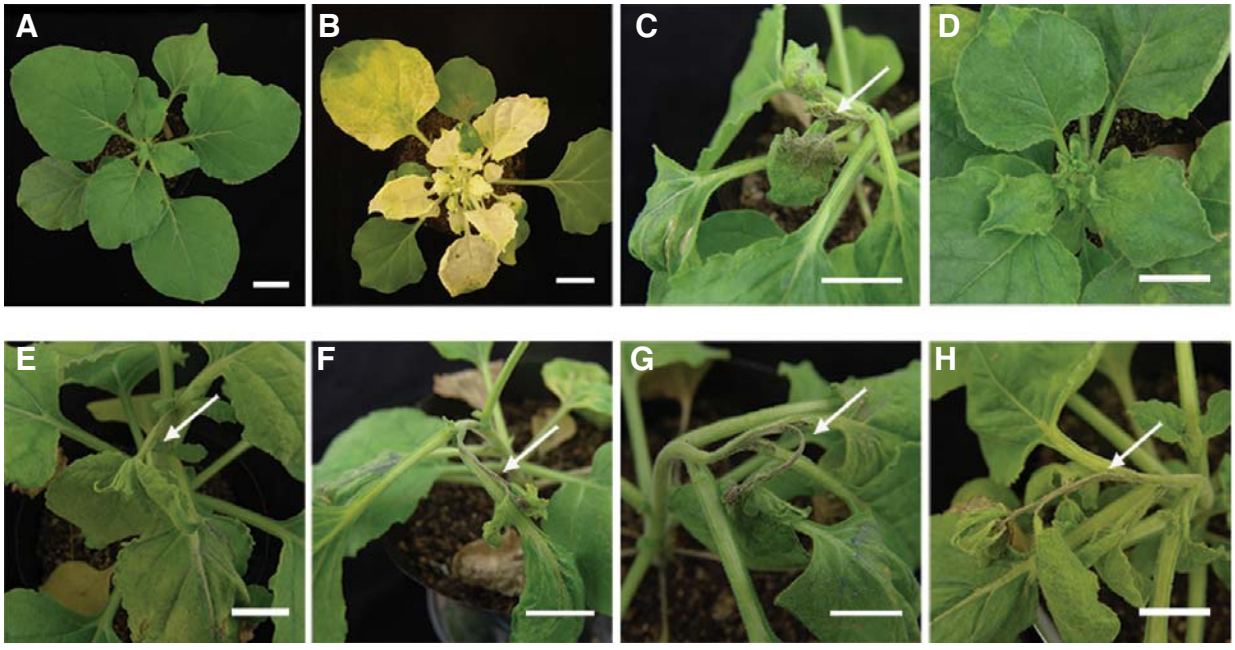

Fig. 6. The effects of various defense-related genes on the SA- and SGT/Hsp90/RAR1-mediated signaling pathways on the LSN phenotype in *N. benthamiana*. (A) pPZP211 vector control, (B) *PDS*, (C) GFP, (D) Nb*SGT1*, (E) Nb*HSP90*, (F) Nb*RAR1*, (G) Nb*EDS1*, and (H) Nb*PAL*. Arrows indicate a withered shoot apical meristems, a sign of LSN by *TTR1* in response to TRSV. Scale bars = 2 cm.

Hsp90, RAR1 and SGT1 play essential roles in development and disease resistance triggered by a number of *R* genes in plants (Hubert et al., 2003; Liu et al., 2004; Tai, 2008; Takahashi et al., 2003). But, in some cases, effector triggered immunity (ETI) and SAR requires only GmRAR1 and GmSGT1, but not GmHsp90, suggesting that RAR1- or SGT1 dependent signaling pathway does not always require Hsp90 in disease resistance in Soybean (Fu et al., 2009). This may be the case

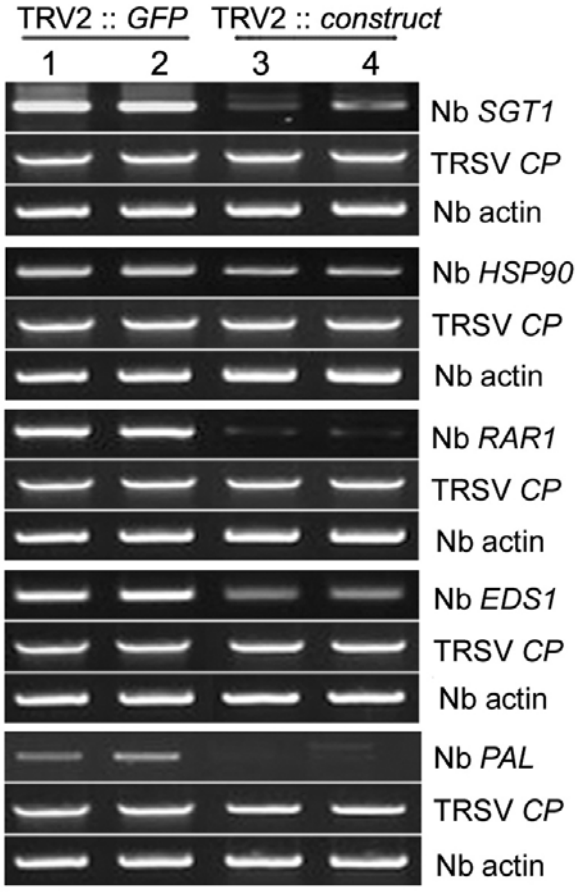

Fig. 7. Semiquantitative RT-PCR analyses to examine endogenous target gene expression levels. At 5 dpi, total RNA was purified from *N. benthamiana* leaf disks and used as a template for RT-PCR (25 cycles) to amplify *N. benthamiana* homologs of various plant defense-related genes to confirm silencing of target mRNAs. The *N. benthamiana* actin mRNA was used as a positive control.

for TTR1 in Est ecotype *Arabdiospsis*, since we found that silencing of *Nb*SGT1 abrogated LSN in TTR1 expressing *N. benthamiana* in response to TRSV infection.

Several lines of evidence have also demonstrated that innate immune responses are conserved in plants and animals (Burch-Smith et al., 2007). Many reports have shown that the Nod protein is a member of a family that includes the apoptosis regulator APAF1, a mammalian Nod-LRR (NLR) protein (Inohara et al., 1999). Two members of NLR family, Nod1 and Nod2, are reported to function as sensors to recognize distinct substructures of intracellular bacteria, activating diverse signal pathways (da Silva Correia et al., 2007). As the mutations of the LRR region of *ttr1* induced LSN following TRSV infection, mutation of the LRR region of Nod2 is also believed to be responsible for Crohn's disease, a chronic inflammatory disease (Chamaillard et al., 2003; Hugot et al., 2001). Nod1 and Nod2 interact with endogenous SGT1 and HSP90, which are components of the protein degradation and folding machinery, and participate in early resistance gene signaling pathways (Austin et al., 2002; Azevedo et al., 2002). Therefore, the LRR regions of both Nod2 and TTR1 may play similar roles with unique three dimensional structures recruiting unique regulatory systems to respond against pathogens. In light of mammalian Nod proteins, we propose that the *Arabidopsis TTR1* supports the concept that innate immunity is conserved across different kingdoms. LSN phenotype observed in Est ecotype (sensitive to TRSV) should be differentiated from SAR of the Col-0 ecotype (tolerance to TRSV). Further investigation of the involvement of SGT1 in the unique TTR1-mediated disease response mechanism could provide more evidence to support conserved innate immunity across different kingdoms.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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