The Role of TIR-NBS and TIR-X Proteins in Plant Basal Defense Responses[1][W][OA]

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Toll/interleukin receptor (TIR) domain-containing proteins encoded in the Arabidopsis (*Arabidopsis thaliana*) genome include the TIR-nucleotide binding site (TN) and TIR-unknown site/domain (TX) families. We investigated the function of these proteins. Transient overexpression of five *TX* and *TN* genes in tobacco (*Nicotiana benthamiana*) induced chlorosis. This induced chlorosis was dependent on *ENHANCED DISEASE RESISTANCE1*, a dependency conserved in both tobacco and Arabidopsis. Stable overexpression transgenic lines of *TX* and *TN* genes in Arabidopsis produced a variety of phenotypes associated with basal innate immune responses; these were correlated with elevated levels of salicylic acid. The TN protein AtTN10 interacted with the chloroplastic protein phosphoglycerate dehydrogenase in a yeast (*Saccharomyces cerevisiae*) two-hybrid screen; other TX and TN proteins interacted with nucleotide binding-leucine-rich repeat proteins and effector proteins, suggesting that TN proteins might act in guard complexes monitoring pathogen effectors.

Innate immunity is a primary defense mechanism in plants that functions to protect against a variety of biotic stresses (Eitas and Dangl, 2010). The plant basal immune system comprises pattern or pathogen recognition receptors that can recognize a variety of plant pathogens by identifying specific pathogen-associated molecular patterns (PAMPs; Tsuda and Katagiri, 2010). This recognition of PAMPs by plant pattern recognition receptors triggers PAMP-triggered immunity or plant basal immunity (Jones and Dangl, 2006; Zipfel, 2008). Well-known PAMPs or microbe-associated molecular patterns recognized by plants include bacterial flagellin, cold shock proteins, and elongation factor Tu. To suppress PAMP-triggered immunity, plant pathogens secrete an array of virulence factors such as type III effector proteins, while plant resistance (R) proteins function to recognize the effector molecules (Römer et al., 2009; Lewis et al., 2010; Tsuda

and Katagiri, 2010; Zhang et al., 2012). Specific recognition of a pathogen effector by a plant R protein triggers a second type of immune response called effector-triggered immunity, resulting in an incompatible reaction (Qi et al., 2011; Sohn et al., 2012; Tahir et al., 2012).

The most commonly known plant R proteins are the nucleotide-binding (NB) site Leucine-rich repeat (LRR) proteins that plants use to detect effector proteins. The NB is often called NB-ARC because of sequence similarities to the human apoptotic protease-activating factor APAF1 and *Caenorhabditis elegans* homolog CELL DEATH PROTEIN4 (Lukasik and Takken, 2009). Plant NB-LRR proteins often also have, at the N terminus, a Toll/Interleukin-1 receptor (TIR) or coiled coil (CC) domain (Meyers et al., 2003). In animal TIR proteins, this domain is more commonly located at the C terminus and is linked by a transmembrane domain to an N-terminal LRR domain (Torto et al., 2002). In *Drosophila* spp. and other microbes, a TIR domain has been shown to play an important role in the activation of antifungal immune responses (Jenkins and Mansell, 2010). Toll-like receptors (TLRs) perform an integral role in the activation of antimicrobial responses in many animals (Radhakrishnan and Splitter, 2010).

In plants, two additional TIR-containing protein families, TIR-NB site (TN) and TIR-unknown/random (TX), were identified, which are distinct from the longer TIR-NB-LRR (TNL) R protein homologs (Meyers et al., 2002). TN proteins contain TIR and NBS domains but lack LRRs, while TX proteins lack both NBS and LRR domains, yet often have a small and variable C-terminal domain (Meyers et al., 2002). In the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) genome, there are 30 *TX* genes and 21 *TN* genes

(Meyers et al., 2003). The crystal structure of a TIR domain from an Arabidopsis TN protein (At1g72930/NP_177436) contains a compact globular fold resembling the mammalian (TLR1 and MYELOID DIFFERENTIATION PRIMARY RESPONSE GENE88 [MYD88]) and bacterial TIR domain proteins (Chan et al., 2010). Although plant TIR domains share less than 20% sequence identity with the human TLR domains, the structures of the TIR domain in plants, mammalian TLRs, and bacterial TIR domain proteins have strong similarity (Chan et al., 2010).

A high proportion of *TX* and *TN* genes were previously reported to be in complex clusters with *TNL* genes; these clusters were found to be duplicated to multiple locations in the genome (Meyers et al., 2002). The existence of genetically linked pairs or sets of genes such as *RESISTANCE TO PERONOSPORA PARASITICA2A* (*RPP2A*)-*RPP2B*, *RESISTANCE TO PSEUDOMONAS SYRINGAE1* (*RPS1*)-*RPS4*, *LEAF RUST RESISTANCE GENE10* (*LR10*)-*RESISTANCE GENE ANALOGUE2* (*RGA2*), *RICE BLAST RESISTANCE GENE AT PIK LOCUS1* (*PIKM-1*)-*TS-PIKM2-TS*, and *RICE BLAST RESISTANCE GENE AT PI LOCUS1* (*PI5-1*)-*PI5-2* in the genomes and their role in disease resistance suggests that these linked genes are required to effect a defense response in plants (Eitas and Dangl, 2010). The genomic pairing of the *TNL* genes with *TX* or *TN* genes suggests a role of the tightly linked TN protein in the function of its cognate TNL protein or proteins (and vice versa).

The specific direct or indirect interaction between an *R* gene and a corresponding *avirulence* (*Avr*) gene in the characterized pairs of interaction resulted in an immune response in the form of localized programmed cell death, called the hypersensitive response (HR; Burch-Smith et al., 2007; Caplan et al., 2008). The recognition of avirulence proteins from pathogens by the cognate R proteins induces a cascade of changes that increases the levels of salicylic acid (SA), jasmonic acid (JA), phenyl ammonium lyase, and systemin (Liu et al., 2010). The production of several of these biochemical signals is known to trigger multiple convergent 'R'-gene signaling pathways, leading to programmed cell death and further changes in gene expression patterns (Vlot et al., 2008a, 2008b). Structure function analysis of Arabidopsis R proteins RPS4 (Zhang et al., 2004) and RPP1A (Michael Weaver et al., 2006) have shown that TIR and NBS domains of the proteins without the LRR domain (TNL truncations) could be sufficient to induce HR. Studies using overexpression of plant *R* genes (particularly the truncated *TNL* genes) suggest that the TIR and NBS domains by themselves might be sufficient to induce HR and to initiate plant defense responses (Michael Weaver et al., 2006; Swiderski et al., 2009).

In this study, we present experimental and computational data that are collectively consistent with a role for Arabidopsis TX and TN proteins in plant defenses. For example, the ability of the *TX* and *TN* genes to induce HR responses upon transient expression is dependent on *ENHANCED DISEASE RESISTANCE1* (*EDS1*). This *EDS1* dependency in induced HR was demonstrated in both tobacco (*Nicotiana benthamiana*) and in Arabidopsis. Stable transgenic overexpression in Arabidopsis of *TX* and *TN* genes resulted in a variety of phenotypes involved with basal innate immune responses that are dependent on SA. We also demonstrated the interaction of TX and TN proteins with plant pathogenic elicitor proteins and other plant signal transduction proteins.

RESULTS

Phylogeny and Conservation of TX and TN Proteins

Based on a phylogenetic analysis of the TIR protein domain within the Arabidopsis Col-0 genome, TX proteins are distinct from TN proteins (Supplemental Fig. S1; Meyers et al., 2002). This is consistent with additional features distinct from TNL plant R proteins such as a lack of NBS domain sequences and or the presence of variable domains at their C terminus. The N-terminal TIR domain is conserved among all three groups. The conserved motifs in the NBS region of the TN protein sequences were shown to be consistent with motifs in the NBS region of the TNL proteins, except for the total absence of a conserved domain within the NBS known as RNBS-A motifs (Meyers et al., 2002). Though TX and TN proteins are diversified in their C-terminal domains, they are more closely related and conserved in their TIR domain regions compared with TNL proteins. Two TN proteins, AtTN17 and AtTN21 (Supplemental Fig. S1 contains a key to the shorthand identifiers), uniquely possess unusual domains at their N and C termini, and thus they have been identified as "XTNX" proteins (Meyers et al., 2002). The phylogenetic analysis demonstrated that these two proteins are outliers in the TX and TN phylogeny, consistent with our prior analysis (Meyers et al., 2002). The X domain in the TX and XTNX proteins was comprised of a variety of diverse sequences ranging in length from 28 to 235 amino acids that were either unique or similar to other TX proteins. Among other variations in their structures, *TX* and *TN* genes differ substantially in the number of introns, with *TN* genes (16 out of 21) comprised of two exons with a single intron, while the *TX* genes typically included more than one intron, which are more often intertwined into X (unknown) domains (Supplemental Fig. S1).

We next examined the conservation of the TX, TN, and XTNX proteins using plant genomic sequences from GenBank. To identify homologs, we used Arabidopsis TN (AtTN3, AtTN10, and AtTN11) and TX (AtTX21) protein sequences plus the two outlying XTNX proteins AtTN17 and AtTN21 for this analysis. Five of the TN proteins yielded protein homologs in a BLAST search against sequences from monocots, basal angiosperms, and magnoliids (Supplemental Table S1). Arabidopsis coiled coil-NBS-LRR (CNL) and TNL reference
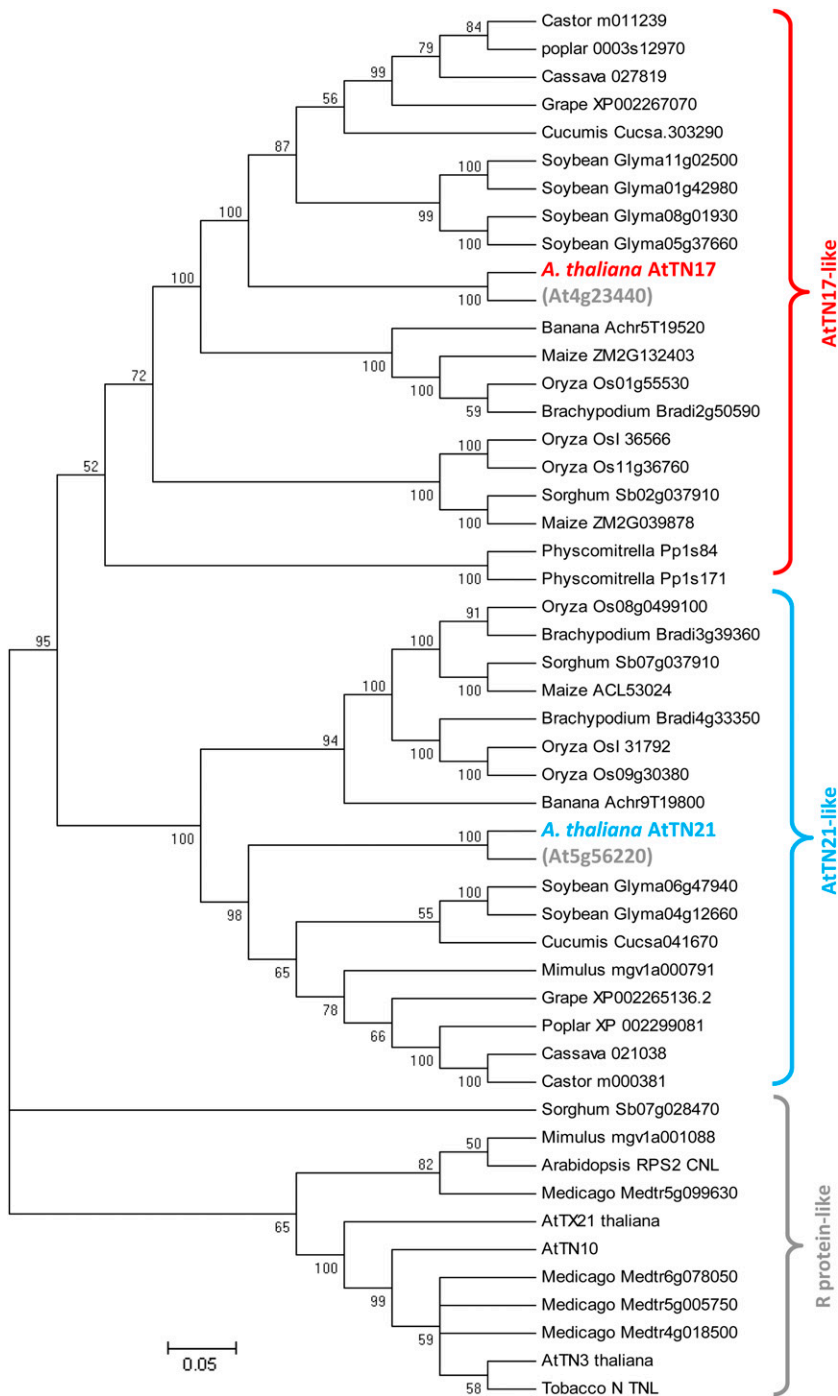
Figure 1. Phylogeny and conservation of TX and TN protein domains. A neighbor-joining phylogenetic tree shows the homology of TN sequences across plants. Arabidopsis TN proteins AtTN21 and AtTN17 (highlighted in color) were used as template sequences to compare against plant protein databases. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 50 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,556 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

proteins such as RESISTANCE TO *PSEUDOMONAS SYRINGAE1* (*RPM1*) and RPP1 displayed a broad range of homology to all the members identified from lower monocots and gymnosperms (Supplemental Table S1). Thirty-five different homologs of AtTN17 and AtTN21 were found in rice (*Oryza sativa*), grape (*Vitis vinifera*), soybean (*Glycine max*), poplar (*Populus* spp.), *Sorghum* spp., *Physcomitrella* spp., castor bean (*Ricinus communis*), maize (*Zea mays*), cassava (*Manihot esculenta* Crantz.), *Cucumis* spp., papaya (*Carica papaya*),

Mimulus spp., *Brachypodium* spp., and banana (*Musa* spp.; Fig. 1), indicating that these two proteins are conserved among diverse plant species. These matches exceeded 60% identity for 41 of the proteins, demonstrating that the XTNX clade is a highly conserved group across all plants in which homologs were found. In rice, three homologs (*LOC_Os01g55530*, *LOC_Os08g38970*, and *LOC_Os09g30380*) of the Arabidopsis XTNX proteins AtTN21 or AtTN17 were identified. Similarly, in banana (a nongrass monocot), two homologs (Achr9T19800_001

and Achr5T19520_001) of the Arabidopsis XTNX proteins AtTN21 or AtTN17 were identified. The rice XTNX homologs *LOC_Os08g38970* and *LOC_Os01g55530* are moderately expressed (Supplemental Table S2). The two XTNX proteins were found to have highest percentage identity (60%–70%) among basal angiosperms (*Amborella trichopoda*, *Aristolochia fimbriata*, and *Nuphar advena*), monocots (*Asparagus officinalis* and *Musa acuminata*), and magnoliids (*Persea americana*, *Saruma henryi*, and *Liriodendron tulipifera*). The other TN proteins that have homologs in *Amborella trichopoda*, *Nuphar advena*, *Liriodendron tulipifera*, and *M. acuminata* were AtTN3, AtTN10, and AtTN11 (Supplemental Table S1). In banana, a single additional TX protein (GSMUA_AChr9G24500_001, 59% identity to At1g61105/AtTX9 and At1g52900/AtTX3) was identified, the first *TIR*-encoding gene other than a *XTNX* identified in a monocot. This banana protein has 55% to 60% overall identity to the Arabidopsis TIR protein (TIR motif from TX and TNL proteins).

Expression of TX and TN Genes in Arabidopsis

The pattern of expression of *TX* and *TN* genes may provide clues to their cellular roles. Our prior work demonstrated expression of *TX* and *TN* genes in EXPRESSED SEQUENCE TAGS (EST) or massively parallel signature sequences data sets (Meyers et al., 2002). With the availability of much larger data sets in Arabidopsis, such as microarray or Illumina-based sequencing-by-synthesis (SBS) data sets, we examined expression of the *TX* and *TN* genes under a variety of conditions, including biotic stress based on microarray data sets available from Genevestigator (Supplemental Fig. S2). *TX* and *TN* genes in Arabidopsis were found to be induced under biotic stress conditions as expressed in microarray data sets (Supplemental Fig. S2). Some of the *TN* genes (*AtTN10*, *AtTN11*, *AtTN2*, and *AtTN3*) showed a higher fold induction in their expression under biotic stress treatments, including infection by *Blumeria graminis*, *Erysiphe cichoracearum*, and *Pseudomonas syringae* infection (Supplemental Fig. S2). The expression of *TX* and *TN* genes in 17 SBS libraries of Arabidopsis tissues

Table I. *Expression of TX and TN genes in Arabidopsis, detected in public SBS data sets*

TX/TN Gene	Annotation	Signature Abundance[a]	No. of Libraries in which Gene Is Expressed[b]	Leaf Control	SA Induction (52 h)
		Transcripts per million			
At1g17610	AtTN1	4–103	3	4	103
At1g47370	AtTX1	3–19	5	0	0
At1g65390	AtTX10	2–6	4	2	0
At1g66090	AtTN3	1–21	5	0	1
At1g72850	AtTN4	1–25	10	4	7
At1g72870	AtTN5	1–8	6	0	0
At1g72890	AtTN6	2–191	14	20	13
At1g72900	AtTN7	3–1,382	10	199	805
At1g72910	AtTN8	9–15	2	15	9
At1g72920	AtTN9	2–26	6	0	0
At1g72930	AtTN10	1–1,499	17	369	1,499
At1g72940	AtTN11	3–215	15	93	117
At1g72950	AtTN12	2–30	7	0	0
At2g03300	AtTX12	0	0	0	0
At2g20145	AtTX13	2–79	7	4	12
At2g32140	AtTX14	4	1	4	0
At3g04210	AtTN13	6–2,008	14	654	2,008
At4g04110	AtTN14	0	0	0	0
At4g09420	AtTN15	2–76	6	2	0
At4g11340	AtTX15	0	0	0	0
At4g16990	AtTN16	1–476	8	90	20
At4g19920	AtTX17	4–112	4	0	0
At4g23440	AtTN17	1–58	15	38	1
At4g23513	AtTX21	15–132	3	0	0
At5g44920	AtTX25	1–695	14	0	30
At5g45000	AtTX26	0	0	0	0
At5g45070	AtTX27	8–150	3	0	0
At5g45080	AtTX28	22–32	2	0	0
At5g45090	AtTX29	2–4	2	0	0
At5g45220	AtTX30	12–13	3	0	0
At5g46480	AtTN19	0	0	0	0
At5g48780	AtTN20	1–5	5	0	0
At5g56220	AtTN21	9–49	14	9	0

[a]Ranges in different libraries indicated in transcripts per million representing maximum and minimum levels. [b]The number with detectable expression out of the 17 mRNA libraries available at http://mpss.udel.edu/at.
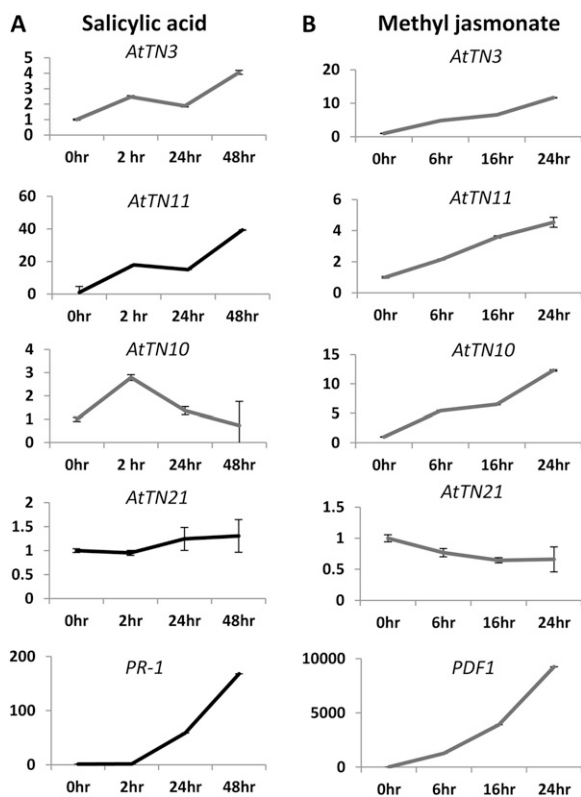
Figure 2. Expression of *TN* and *TX* genes in Arabidopsis Col-0 plants after SA and MeJA treatments. The values on the *y* axis represent fold change of expression at corresponding time intervals on the *x* axis. A, Expression of *TN* and *TX* genes that were chosen based on SBS data from SA-treated libraries, measured by real-time PCR following SA-treatments. The *PR-1* marker gene known to be induced by SA was used as a control (bottom). B, Expression level of *TN* and *TX* genes as measured by real-time PCR experiment following MeJA treatments. The *PDF1* marker gene known to be induced by MeJA was used as a control (bottom).

was also analyzed (Table I; Supplemental Table S3). *AtTN10* and *AtTN13* had the highest and most ubiquitous expression. After SA treatments, five of the *TN* genes (*AtTN1*, *AtTN7*, *AtTN10*, *AtTN11*, and *AtTN13*) were induced by at least 10-fold compared with their expression in the leaf libraries either at 4 or 52 h after treatment (Supplemental Table S3). We checked by quantitative reverse transcription (RT)-PCR the expression levels of four different *TN* genes (*AtTN3*, *AtTN10*, *AtTN11*, and *AtTN21*) in SA-treated plant tissues (Fig. 2). Among the *TN* genes tested, *AtTN3* and *AtTN11* displayed an elevated expression at 48 h compared with the zero-hour time points after SA treatments, which are in agreement with the massively parallel signature sequences data (Fig. 2A; Supplemental Table S3). The highly conserved Arabidopsis gene *AtTN21* was found to have relatively stable expression at different time points, as the gene expression level was relatively unaffected by SA treatments (Fig. 2A). Quantitative RT-PCR measurements also recapitulated SA-induced expression of the genes *AtTN10* and *AtTN11* found in public databases such as Genevestigator and public SBS libraries

(Fig. 2; Supplemental Table S3). The marker gene *PATHOGENESIS-RELATED PROTEIN1* (*PR-1*) (control) was substantially induced with the exogenous application of SA (Fig. 2A). This induction suggests that *TX* and *TN* genes might play a role in SA-mediated defense signaling or responses. In agreement with the microarray data, following the methyl jasmonate (MeJA) treatment, the genes *AtTN3*, *AtTN10*, and *AtTN11* were increasingly induced from 6 to 24 h after treatment (Fig. 2B; Supplemental Fig. S2). Expression levels were 10- to 15-fold higher in *AtTN3* and *AtTN11* compared with its basal levels in Col-0 following the treatment of 10 μM MeJA treatments (Fig. 2B). The marker gene *PATHOGEN-INDUCIBLE PLANT DEFENSIN1.2* (*PDF1.2*; the control) was substantially induced with the exogenous application of MeJA (Fig. 2B). Thus, these *TN* genes are responsive to external application of JA and could act in JA-dependent plant pathways. In summary, the expression patterns of several *TX* and *TN* genes are consistent with roles in or downstream of plant defense pathways.

Transient Overexpression of the TX and TN Genes in Tobacco Results in Cell Death Responses

We next sought to investigate the potential function of these genes using transient assays, knowing that transient overexpression of *R* genes may induce spontaneous HR (Swiderski et al., 2009). Transient *Agrobacterium tumefaciens*-mediated overexpression of Arabidopsis TX and TN proteins in tobacco resulted in HR, a cell death phenotype, for five (*AtTN3*, *AtTN10*, *AtTN11*, *AtTN21*, and *AtTX21*) out of 15 *TX* and *TN* genes tested (Supplemental Table S4). HR cell death was scored based on a relative scoring method using a system of multiple plus signs as the strength increases (Fig. 3A). The cell death phenotype observed for *AtTN3* (+++ = 3) was stronger over the other four transient overexpression events (*AtTN10*, *AtTN11*, *AtTN21*, and *AtTX21*) that resulted in a mild HR response (++ = 2) in tobacco (Fig. 3A). The other 10 *TX* and *TN* genes tested with transient overexpression in tobacco did not display any visible phenotypes (Supplemental Table S5). *AtTN3* induced a more robust cell death response (++++ = 4) when transiently overexpressed together with the exogenous addition of the elicitors SA or ethephon (Fig. 3B). This enhanced cell death was even more apparent when *AtTN3* was transiently overexpressed in conjunction with exogenously applied flagellin (flg22) peptide. The enhanced cell death effect observed by transient overexpression of AtTN3 with the flg22 peptide is possibly a synergistic mechanism in tobacco (Fig. 3B). There was no enhancement effect of these exogenously applied elicitors on the ability of the other 14 *TX* or *TN* genes to induce cell death in tobacco leaves. Therefore, some members of the TX and TN protein family can trigger cell death via ectopic expression, similar to previous reports that used N-terminal domains of the *TNL* genes *RPS4* and *RPP1A* (Zhang et al., 2004; Michael Weaver et al., 2006).

Figure 3. Transient overexpression of Arabidopsis *TN* and *TX* genes in tobacco results in partial cell death response. A, Transient overexpression of *AtTN3* in tobacco results in cell death. Visual scoring of HR responses and HR strength score table (++++ = 4; +++ = 3; ++ = 2; and + = 1) indicate the strength of HR observed with the transient overexpression of other TX and *TN* genes compared with the *TN* gene *AtTN3*. B, Transient overexpression of AtTN3 along with elicitors SA, ethephon, and flagellin results in increased cell death responses in tobacco. C, Transient overexpression of *AtTN3* in an *eds1*-silenced plant along with or without flagellin resulted in a decreased HR in tobacco. Expression of *EDS1* was checked in tobacco plants in both empty vector transformed lines (EV) and *EDS1*-silenced lines (SL) by quantitative RT-PCR.

Transient overexpression of *TX/TN* gene	Strength of HR observed
AtTN3	+++ (3)
AtTN10	++ (2)
AtTN11	++ (2)
AtTN21	++ (2)
AtTX21	++ (2)
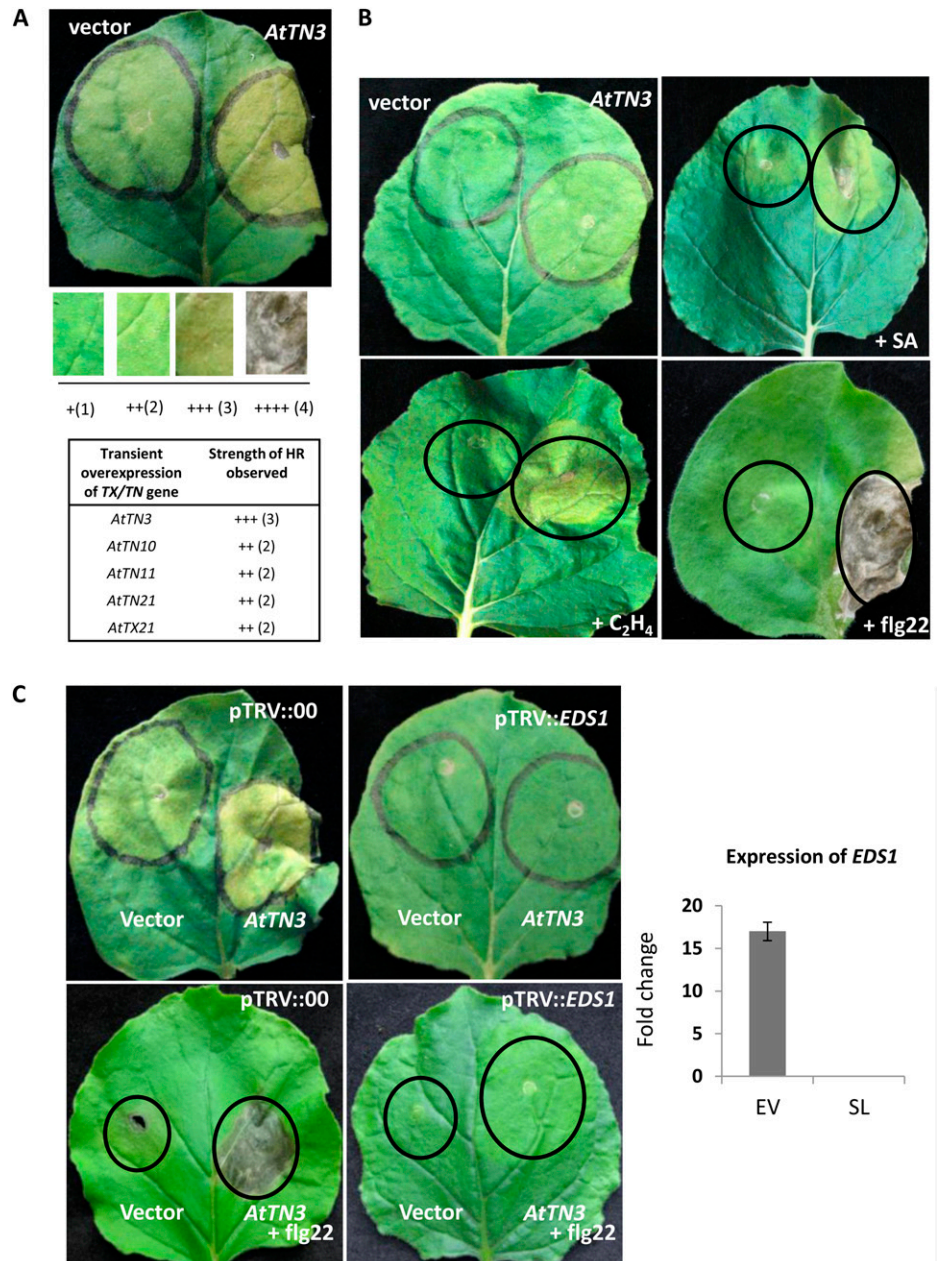
Dependency of TX and TN Genes on EDS1 to Induce Cell Death in Tobacco

Plant TIR domain-containing proteins are reported to signal via pathway that includes EDS1 and PHYTOALEXIN DEFICIENT4 (PAD4) (Nishimura and Dangl, 2010). To investigate the importance of *EDS1* downstream of *TX* and *TN* proteins, transient overexpression of *TX* and *TN* genes was tested in tobacco transiently silenced for *EDS1* by viral-induced gene silencing (VIGS). Transient overexpression of the gene *AtTN3* induced partial cell death of the leaf tissue in the plants inoculated with the empty viral vector. However, *AtTN3*-induced partial cell death was lost in the lines silenced for *EDS1* (Fig. 3C).

Because flg22 can induce defense response genes and trigger responses to plant pathogenic bacteria (Zipfel et al., 2004; Zipfel, 2008), transient overexpression of *AtTN3* gene was tested along with flg22 in both *EDS1*-silenced and nonsilenced plants. In the *EDS1* nonsilenced plant, coinfiltration of *AtTN3* and flg22 was able to cause cell death (Fig. 3C). However, in the *EDS1*-silenced plants, cell death was abolished after coinfiltration (Fig. 3C). Quantitative real-time PCR analysis of both the empty VIGS vector-transformed and *EDS1*-silencing plants showed that expression of the *EDS1* gene was greater than 15-fold higher in the nonsilenced versus silenced plants (Fig. 3C). The results were replicated over five different subsets of plants. Thus, signaling by AtTN3 that results

in cell death in transient overexpression experiments appears dependent on EDS1.

Role of TX and TN Genes in Plant Defense Responses

Induction of plant defense responses occurs upon overexpression of *R* genes (such as *Suppressor of NONEXPRESSOR OF PR GENES1* (*NPR1*) *Constitutive1* [*SNC1*]) in Arabidopsis (Li et al., 2010). Consistent with this, *A. tumefaciens*-mediated transient overexpression of five *TN* and *TX* genes, *AtTN3*, *AtTN10*, *AtTN11*, *AtTN21*, and *AtTX21*, in tobacco triggered cell death. Encouraged by the transient studies in tobacco described above, we assessed the functions of TX and TN genes in Arabidopsis. Initially, knockouts of the *TX* and *TN* genes were screened for visible phenotypes and for susceptibility to plant pathogens (*Albugo*

candida, *Tobacco Mosaic Virus* (*TMV*), avirulent *P. syringae*, and *Hyaloperonospora parasitica*). No visible phenotype was observed for the transfer DNA lines analyzed (data not shown). Lack of an obvious phenotype in Arabidopsis knockouts prompted us to generate stable overexpression lines for *TX* and *TN* genes in Arabidopsis. We generated transgenic lines of Arabidopsis Col-0 with stable overexpression of *TN* and *TX* genes (*AtTN1* to *AtTN4*, *AtTN10*, *AtTN11*, *AtTN13*, *AtTN15*, *AtTN17* to *AtTN19*, *AtTN21*, *AtTX6* to *AtTX10*, *AtTX14*, *AtTX16*, *AtTX21*, *AtTX24*, *AtTX26*, and pK2GW7 as a control) to observe their phenotypic effects (Supplemental Table S6). One of the *TN* genes, *AtTN3*, did not yield any transgenics from transformation into a Col-0 background, potentially due to deleterious effects of the overexpressed transgene. However, we did obtain overexpression lines for *AtTN3* in an Arabidopsis *eds1* background.
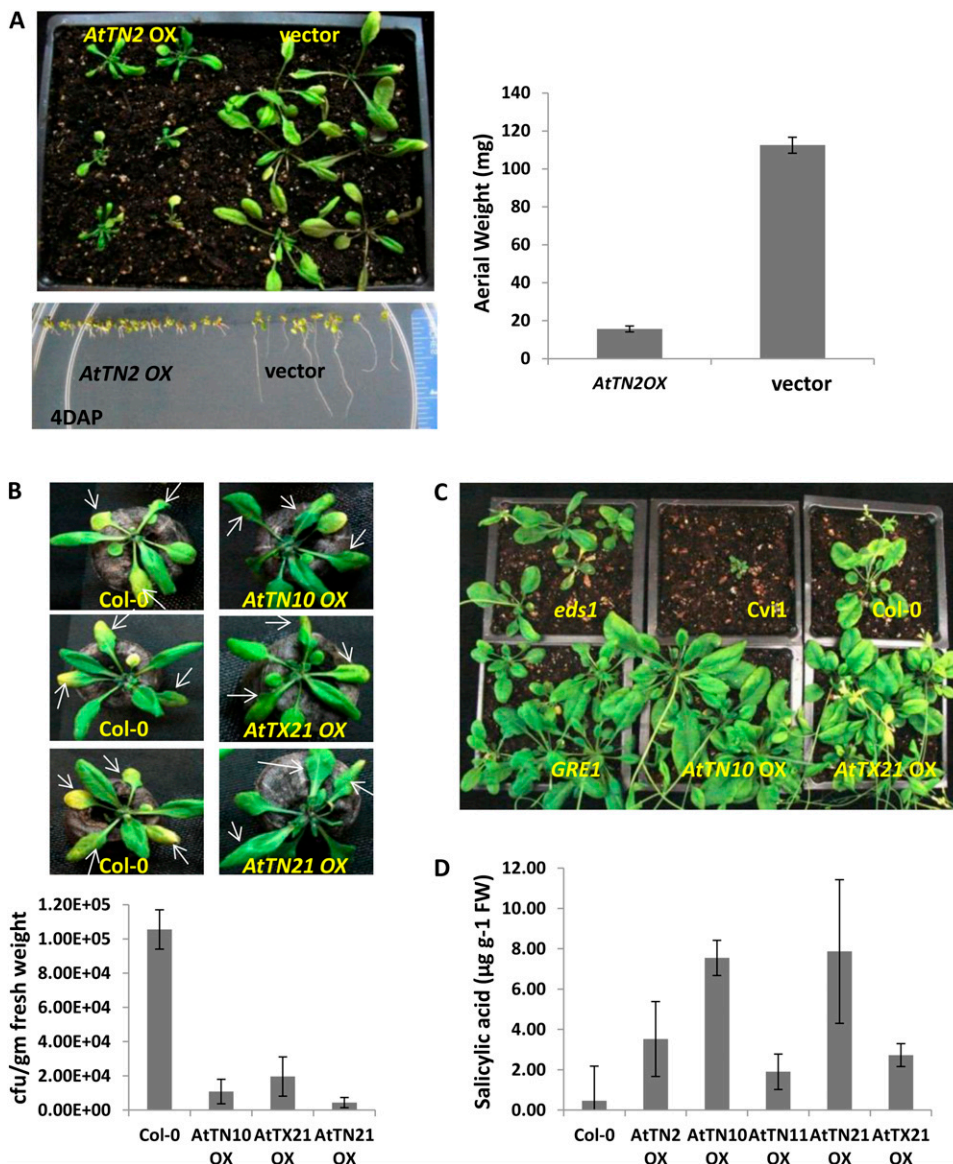

Figure 4. Stable transgenics of *TN* and *TX* genes in Arabidopsis Col-0 plants display plant defense responses. A, Stunted phenotype of overexpression (OX) transgenics of *AtTN2* gene. B, *TX* and *TN* overexpression transgenics showing resistance to virulent bacterial pathogen *Pst* DC3000. Bacterial colony count numbers expressed as colony forming units (CFUs) per gram fresh weight of leaf infected shows lower colony forming units in overexpression transgenics *AtTN10 OX*, *AtTN21 OX*, and *AtTX21 OX*. C, *TN* and *TX* overexpression transgenics showing resistance to *F. oxysporum* strain O-685. D, HPLC analysis of stable *TX* and *TN* overexpression transgenics measuring free SA levels in plants.

All of the overexpression lines, except for *AtTN3*, in Col-0 background were analyzed for developmental phenotypes and tested for response phenotypes to a set of bacterial and fungal plant pathogens. Out of 22 different overexpression stable transgenic lines developed for different *TX* and *TN* genes (Supplemental Table S6), one of the overexpression transgenics, *AtTN2*, displayed stunted growth and developmental defects, both on soil and in plates, and had less aerial weight (Fig. 4A), consistent with phenotypes for *AtTN2* overexpression published by other labs (Li et al., 2009). Lines overexpressing *AtTN10*, *AtTN21*, and *AtTX21* displayed reduced disease symptoms along with lower bacterial titers compared with the wild type, when challenged with virulent bacterial plant pathogen *Pseudomonas syringae* pv *tomato* DC3000 (Pst DC3000; Fig. 4B).

Because overexpression of *NPR1* in Arabidopsis can confer broad-spectrum resistance to the necrotrophic fungal pathogen *Fusarium oxysporum* (Parkhi et al., 2010), we investigated whether *TX*- and *TN*-overexpressing transgenic lines might similarly affect resistance to *F. oxysporum*. The roots of the stable overexpression lines (*AtTN10*, *AtTN11*, *AtTN21*, and *AtTX21*) were inoculated with *F. oxysporum* strain O-685. *AtTN10* and *AtTX21* lines showed increased resistance to the pathogen (Fig. 4C). Upon further analysis of the lines, they showed an increase in SA levels (free SA levels) compared with the control samples (Fig. 4D). Increased SA levels (free SA) could potentially prime systemic acquired resistance (SAR) and other defense responses leading to resistance in these transgenics. We concluded that stable overexpression in Arabidopsis of several *TX* and *TN* genes resulted in increased resistance to bacterial (*Pst* DC3000) and fungal (*F. oxysporum* strain O-685) pathogens.

Subcellular Localization and Interactions of TX and TN Proteins with Effector Proteins Indicate Their Role in Plant Defense Signal Transduction Pathway

We next examined the subcellular localization of TX and TN proteins by transient expression as GFP fusion proteins under the 35S promoter in tobacco. AtTN3 and AtTN10 both demonstrated nuclear and cytoplasmic localization, while AtTN11 and AtTN21 were plasma membrane localized (Fig. 5A). The expression of the fusion proteins AtTN3 and AtTN10 was measured using anti-GFP antibody in a Western blot, and they both were found to have predicted protein fusion sizes, which were 48 and 76 kD, respectively (Supplemental Fig. S3). The membrane localization of AtTN11 and AtTN21 was confirmed using FM4-64 FX staining (Fig. 5A). The transient overexpression of AtTX21 resulted in the formation of cytoplasmic aggregates (Fig. 5A). Thus, TN and TX proteins were localized to distinct, diverse cellular locations, suggesting diverse roles for these proteins.

To identify potential signal transduction proteins and effector proteins that could interact with TX and TN proteins, given that R proteins are known to
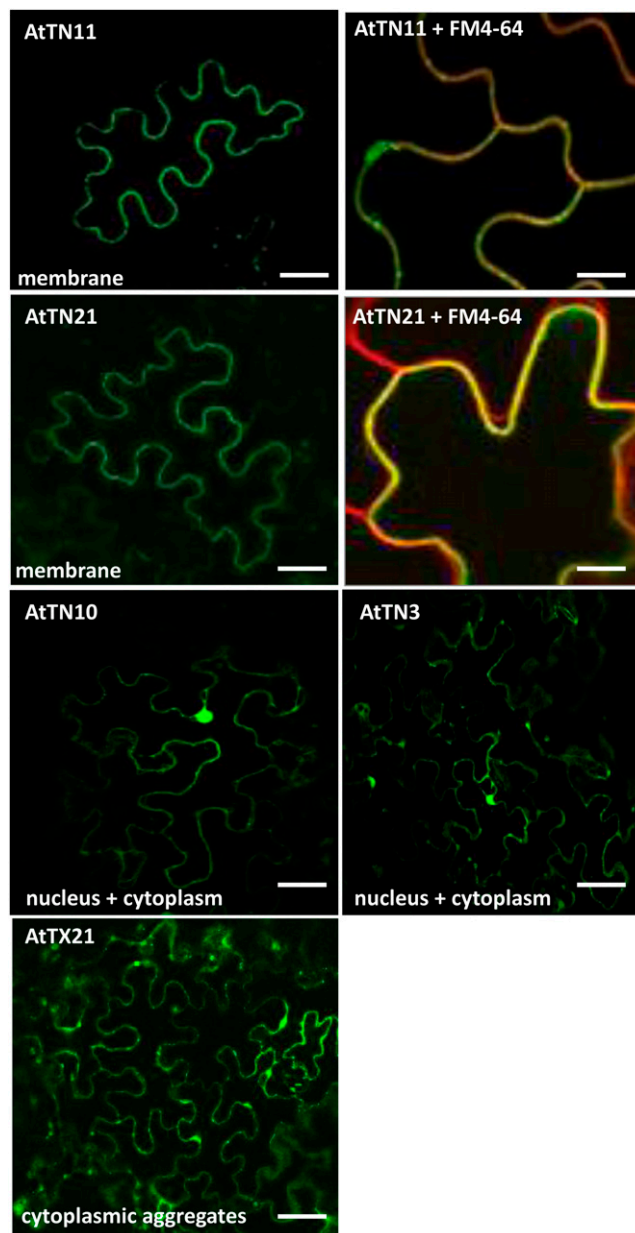

Figure 5. Transient overexpression of TN and TX proteins in tobacco leaves. AtTN11 and AtTN21 proteins show membrane localization, observed through FM4-64 staining in the insets. AtTN11 and AtTN21 proteins are showed in green and FM4-64 staining in red. The colocalization of FM4-64 and Arabidopsis membrane-localizing TN proteins is shown in yellow. AtTN10 and AtTN3 show nuclear and cytoplasmic localization pattern in tobacco. The localization of AtTN10 and AtTN10 is shown in green. Transient overexpression of AtTX21 shows cytoplasmic aggregates and cytoplasmic localization. The localization of AtTX21 was shown in green. Bars = 20 μm.

interact with plant signal transduction proteins (Eitas and Dangl, 2010), we performed a yeast (*Saccharomyces cerevisiae*) two-hybrid (Y2H) experiment with known and candidate effector targets, components of NB-LRR proteins. A subset of the TX and TN proteins (AtTN1, AtTN3, AtTN10, AtTN11, AtTN15, AtTN21, AtTX6,

Table II. *TN and TX proteins interacted with a diverse set of elicitors and NBS-LRR proteins in a Y2H screen*

TX/TN Protein	Effectors and Signal Transduction Proteins Identified as Interacting
AtTN1	Protein kinase (Pto)66 (AvrPto) of *P. syringae* and Ral011t (*R. solanacearum*), *Magnaporthe oryzae* (Mor003)
AtTN3	Pto42 (HopY), Brl028 (*B. Lactucae*), At3g14470 (NB-LRR)
AtTN6	No interactions detected
AtTN7	Ral019 (HopF2; *R. solanacearum*)
AtTN10	Rbp001 (root knot nematode effector), Urf004
AtTN11	Hpp001 (*H. parasitica*)
AtTN13	Pto42 (HopY), Rbp001 (nematode effector)
AtTN15	Psy(2), Pto(8), Ral(4), Rbp(3), Pvx001, Pph(2), Urf003, Urf004; Brl011, Pma005
	Plant proteins: At1g59124, AtACT, Arabidopsis Jasmonate response1, At1g61190, At5g05400, At1g31540
AtTN21	*P. syringae*: Psy(2), Pto(5), Pph(3)
	R. solanacearum: Ral(6)
	Pseudomonas flourescens: Pfl003, *B. lactucae*: Brl(6), *H. parasitica*: Hpp001, Hpp021
	Nematode: Rbp001, Rbp002, Mor003; Pvx001
	Plant proteins: At5g63020 (NB-LRR), At1g12210, At4g16950, At4g36150, At5g63020, AtACT, AtEDS1, AtPBS1, AtTGA1, AtTGA2, AtTGA3, AtTGA7, AtMPK4, LeCMPG1, AtTN21
AtTX6	No interactions detected
AtTX8	Ral019 (HopF2; *R. solanacearum*)
AtTX16	(Psy022, psy023), Pto(4), Ral025, Pph022, Mor003; plant proteins: At3g14470, AtACT, AtTGA3, Arabidopsis MAP Kinase4, LePto
AtTX21	Pto66 (AvrPto) of *P. syringae*

AtTX8, AtTX16, and AtTX21) was chosen based on their phenotypes in earlier experiments (transient and overexpression studies). The representative class of TX and TN proteins screened against plant pathogenic effectors displayed interactions with bacterial, fungal, and nematode effector proteins (Table II). Based on the number of interactions with TX and TN proteins and the strength of interactions, mapping was done to describe the interactions along with their strength index (Supplemental Fig. S4). Among the TX and TN proteins that were chosen as baits, AtTN21 has the highest number of interactions with candidate elicitor proteins from *P. syringae*, *Ralstonia solanacearum*, *Bremia lactucae*, and *Hyaloperonospora arabidopsidis*. Among other notable interactions, few plant proteins (AtEDS1 and Arabidopsis serine/threonine Protein Kinase PBS1; AtPBS1) that are involved in disease resistance pathways were found to interact with AtTN21. Similarly, AtTN15 was found to interact with effectors from *P. syringae*, *R. solanacearum*, *B. lactucae*, and *H. arabidopsidis* as well as plant NBS-LRR proteins (Supplemental Fig. S4; Table II). Two of the TN proteins, AtTN3 and AtTN21, interacted with components of NB-LRR proteins, At3g14470 and At5g60320, respectively. The Arabidopsis protein At3g14470 is an RPP13-like NB-LRR protein, while At5g60320 is a lectin kinase-like protein. Therefore, TN and TX proteins may function with other proteins involved in plant disease resistance pathways.

Arabidopsis TN Protein AtTN10 Interacts in the Cytoplasm with a Chloroplastic Protein, 3-Phosphoglycerate Dehydrogenase

Next, we sought to expend the set of cellular partners with which TX or TN proteins might function. The TN protein AtTN10 was selected for analysis as a bait protein for screening a Y2H library of prey proteins constructed from mRNA derived from several growth stages of Arabidopsis. Strong interactions were found between AtTN10 and 3-phosphoglycerate dehydrogenase (3PGDH, At1g17745), a chloroplastic protein. We used a colocalization assay to assess their interaction in vivo. When transiently coexpressed in epidermal cells of tobacco, the two proteins AtTN10 and 3PGDH both colocalized in the cytoplasm (Fig. 6A). Additional control experiments that involved the localization of AtTN10 with cyan fluorescent protein (CFP) nonfusion protein helped us to confirm the nuclear exclusion interaction of 3PGDH with AtTN10 (Fig. 6A; Supplemental Fig. S5). The sequence comparison of 3PGDH with orthologous proteins from other plant species shows the highly conserved nature of the protein (Supplemental Fig. S6). A search of available expression data from Genevestigator showed the induced expression (more than 2.5 times) of the gene *3PGDH* in Col-0 plants infected by plant pathogens such as *P. syringae*, *B. graminis*, *Botrytis cinerea*, and *Alternaria brassicola*. Transient overexpression of 3PGDH-tagged CFP fusion protein was found to localize in the subcellular compartments of chloroplasts along with cytoplasm (Fig. 6A).

To understand whether AtTN10 might be playing a role as an R protein, we transiently overexpressed AtTN10 in a transgenic background with N RECEPTOR-INTERACTING PROTEIN1 (NRIP1) cerulean fusion protein (which fluoresces blue). The TNL protein N of tobacco was shown to interact with p50 domain of TMV through its TIR domain (Burch-Smith et al., 2007). NRIP1 was recently identified to interact with both the TIR domain of N and the p50 domain of TMV (Caplan et al., 2008). NRIP1 transgenics were previously generated by expressing NRIP1 tagged with C-terminal cerulean under the control of the *NRIP1* genomic promoter (Caplan et al., 2008), and NRIP1 has
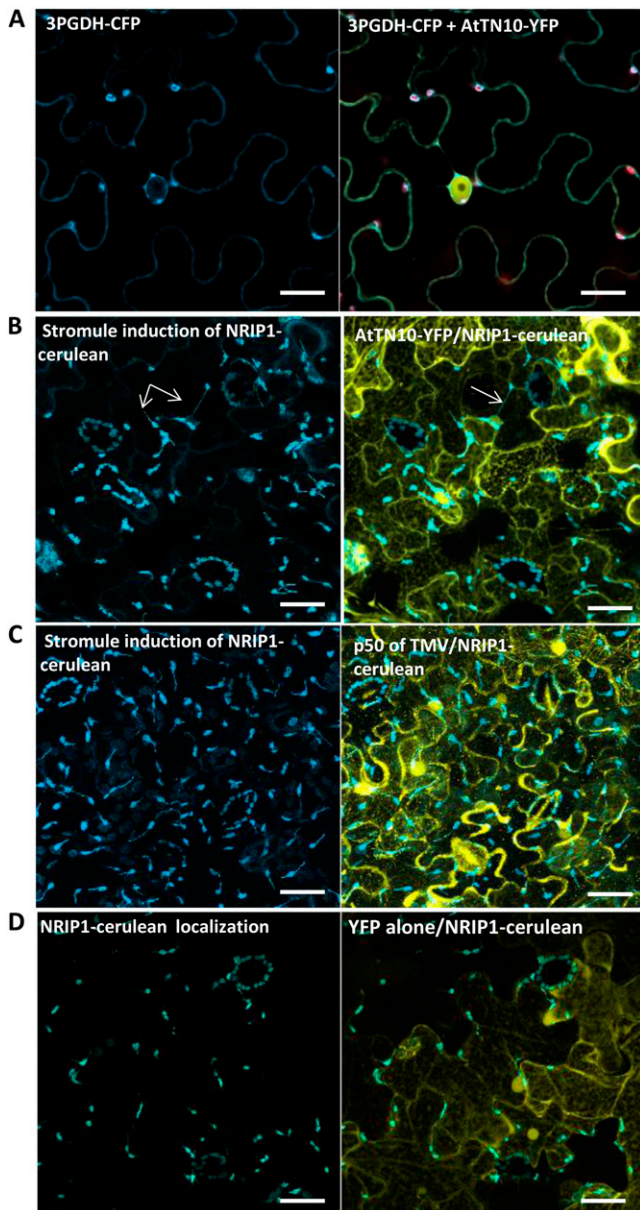
Figure 6. Arabidopsis TN protein AtTN10 interacts with 3PGDH in the cytoplasm. A, AtTN10 transiently cooverexpressed with 3PGDH in tobacco leaves results in cytoplasmic localization. B, AtTN10 induces strong stromule formation, as observed by the NRIP1 in chloroplasts when AtTN10 is transiently overexpressed in NRIP1 transgenic background. C, p50 protein of TMV induces strong stromule formation in NRIP1 background. D, Negative control showing YFP protein alone transiently overexpressed in NRIP1. Stromule induction is shown by white arrows. Bars = 20 μm.

been shown to localize in the chloroplasts (Caplan et al., 2008). Transient overexpression of AtTN10 in the NRIP1 background resulted in a strong stromule induction of NRIP1 (Fig. 6B), comparable to the stromule induction by p50 protein of TMV in NRIP1 (Fig. 6C). Stromule induction in the NRIP1 background appeared to require AtTN10, as the nonfusion yellow fluorescent protein

(YFP) control protein did not induce this phenotype (Fig. 6D). Therefore, AtTN10 overexpression can induce a cellular phenotype (stromule formation) similar to a TMV-triggered immune response.

DISCUSSION

Plant TX- and TN-encoding genes appear to be largely restricted to dicot genomes that parallel the taxonomic distribution of the related *TNL* R genes. TN proteins were found to be conserved among many diverse plant genomes, including gymnosperms, basal angiosperms, monocots, and magnoliids. Two divergent XTNX proteins are highly conserved, apparently in many flowering plants, suggesting an ancient and important role of these proteins. The presence of a single *TN*-like *TIR* gene in the recently sequenced *M. acuminata* genome (a nongrass monocot) indicates that *TIR* genes have been largely but not completely eliminated from monocot genomes (*XTNX* genes excluded); the banana TX gene may be a vestige of larger numbers of such genes in earlier plants. The specific functions of the larger set of TX and TN plant proteins remain unclear, but we present evidence consistent with their function in defense against pathogens (Supplemental Table S7). The expression of few of the *TN* genes in the publicly available microarray databases and the SBS databases under different biotic treatments suggests the possibility of their role in plant defenses. Furthermore, the effect of exogenously supplied SA resulted in the induction of two *TN* genes, *AtTN3* and *AtTN11* in Arabidopsis Col-0 plants. Interestingly, similar effect of induction was observed with three Arabidopsis *TN* genes, *AtTN3*, *AtTN10*, and *AtTN11*, when MeJA was applied. Evidence from previous reports suggests a reciprocal antagonism between two pathways, SA and JA (Thaler et al., 2012). The transcriptional induction of two TN genes *AtTN3* and *AtTN11* in both the applications of SA and JA could be due to their position at the convergence of these two pathways or might be due to their interference with the SA-JA cross talk regulators (Verhage et al., 2010).

Overexpression of TX and TN Genes Activates Defense in Both Tobacco (Transient Expression) and Arabidopsis (Stable Transgenic Lines)

We used reverse genetics approaches to study the functions of genes encoding TX and TN proteins in Arabidopsis. Initially, knockouts of the *TX* and *TN* genes were screened for phenotypes, such as loss of resistance or susceptibility to plant pathogens. Lack of an obvious phenotype in Arabidopsis knockouts prompted us to generate transient and stable overexpression lines for *TX* and *TN* genes in tobacco and Arabidopsis. Out of 15 *TX* and *TN* genes tested for transient overexpression in tobacco, four of the *TN* genes and one *TX* gene induced partial cell death responses. Stable overexpression of *TN* and *TX* genes in Arabidopsis resulted in phenotypes consistent with plant defense

responses, including a lethal phenotype (by *AtTN3*), similar to that observed in the leaves of tobacco. Induction of a localized cell death has been demonstrated previously in tobacco when an *R* gene is transiently overexpressed in the absence of a corresponding Avr protein (Swiderski et al., 2009). Cell death induced in response to the transient overexpression of *TNL* genes was shown in the cases of *RPP1A* and *RPS4* (Zhang et al., 2004; Swiderski et al., 2009). Thus, the TIR domain is in common between the TX, TN, and TNL proteins and may have a shared roll in triggering cell death.

Stable transgenic overexpression lines of *AtTN10*, *AtTX21*, and *AtTN21* resulted in resistance to the bacterial plant pathogen *Pst* DC3000 and, in *AtTN10* and *AtTX21*, resistance to the necrotrophic fungal pathogen *F. oxysporum*. *AtTN2*, which caused a stunted phenotype upon stable overexpression, has also been identified as *lesion cell death gene4* (*LCD4*) in a transgenic experiment (Li et al., 2009). The same gene was also identified as a modifier of *SNC1*, which is negatively regulated by *BONZAI1* (*BON1*) and *BON3* (Li et al., 2009). In Arabidopsis, the *BON1/copine1* (*CPN1*) gene in association with *BON2* or *BON3* has been shown to negatively regulate cell death and defense responses (Yang et al., 2006). We found that stable *AtTN2*-expressing transgenic lines displayed a stunted phenotype and had high free SA levels. Reduced plant fitness has also been observed from priming of SA-related defense responses upon *R* gene overexpression in Arabidopsis (including *RPM1*, *Pseudomonas resistance and fenthion sensitivity* (*Prf*), *SNC1*, and *ACCELERATED CELL DEATH6* (*ACD6*); Li et al., 2010; Todesco et al., 2010). Thus, the stable transgenic lines that we obtained overexpressing TX and TN proteins also displayed phenotypes that were phenocopies of overexpression lines for genes involved in plant defenses.

The Dependency of TX and TN Genes on EDS1, a Key Component in the Plant Defense Signaling Pathway

EDS1, PAD4, and NON-RACE-SPECIFIC DISEASE RESISTANCE1 (NDR1) transduce signals in plants upon recognition of plant pathogens (Eitas and Dangl, 2010; Nishimura and Dangl, 2010; Bhattacharjee et al., 2011). We tested the hypothesis that TX and TN proteins might have a similar requirement for EDS1 signaling by transient overexpression of *TX* and *TN* genes in tobacco plants silenced for *EDS1*. As in similar experiments with *RPS4* and *RPP1A* (*TNL* genes; Zhang et al., 2004; Swiderski et al., 2009), cell death caused by the transient overexpression of *AtTN3* gene was dependent on *EDS1*. In Arabidopsis, we were unable to generate any AtTN3 transgenics in a Col-0 background (data not shown), while we were able to develop these in an *eds1* background (Supplemental Table S6). The synergistic cell death effect observed by the transient overexpression of AtTN3 and flg22 peptide in tobacco was also found to be dependent on EDS1 (Fig. 3C).

The dependency of *AtTN3* on EDS1 in heterologous tobacco system demonstrates the requirement of *EDS1* for plant defense responses by *AtTN3*.

Interaction of TX and TN Proteins with Effectors and Plant Defense Signal Transduction Proteins

We tested the interaction of TX and TN with plant signal transduction proteins and pathogen effector proteins using the Y2H system. The protein interaction study resulted in a diverse set of interactions between TX or TN proteins with the candidate effectors tested. The interacting proteins identified through the Y2H experiment (Table II; Fig. 5) suggests the possible role of the TN or TX proteins in recognizing the plant pathogenic effectors either directly or indirectly. One of the XTNX proteins, AtTN21, interacted with several of the candidate effector proteins from at least four different plant pathogens. Intriguingly, AtTN21 was also shown to interact with other plant proteins, including PBS1, a signaling component of CC-NBS-LRR protein RPS5. The interactions of AtTN21 with plant proteins such as PBS1 or effector proteins might have to do with a role as an adapter protein. This is particularly interesting given the level of conservation observed for AtTN21 with homologs in grass and other nongrass genomes. We also searched for interactions of the TX and TN proteins with a broader set of Arabidopsis proteins, and AtTN10 was found to interact with a chloroplastic protein, 3PGDH. More interestingly, colocalization experiments performed by fluorescence tagging of AtTN10 and 3PGDH resulted in both the proteins colocalizing with each other, suggesting that the interaction might occur in the cytoplasm rather than the chloroplasts. Bacterial and viral pathogens are known to target chloroplasts or chloroplastic proteins such as NRIP1 for degradation, reducing host defenses (Caplan et al., 2008). Transient overexpression of *3PGDH* in tobacco leaves showed a chloroplastic and cytoplasmic localization of the protein, whereas expression of AtTN10 in an *NRIP1* transgenic background strongly induced chloroplastic stromule formation. The formation of stromules is induced by the TMV p50 protein during HR in tobacco (Caplan et al., 2008). AtTN10-induced stromules formed in the absence of effector proteins suggests that AtTN10 could function in responses similar to those induced by plant R proteins. It is possible that the 3PGDH protein could be the "guardee," and AtTN10 might play a role as an adapter for this protein, allowing for degradation or modification (Supplemental Fig. S7).

Arabidopsis TX and TN proteins were previously hypothesized to function as adapter proteins in plants similar to MyD88 and MyD88-adapter-like (Mal) adapter proteins in mammalian and *Drosophila* spp. immune responses (Meyers et al., 2002). Here, some of the experimental data reveals a possibility of such a role for TX and TN proteins in the plant defense against plant pathogens (Supplemental Fig. S7). In the model,

we hypothesize that the two classes of TN and TX proteins may function as adapter proteins in plant defense signaling (Supplemental Fig. S7). The interaction of TN proteins with NB-LRR plant proteins and with proteins that are involved in the plant defense response pathways strengthens the hypothesis of these proteins acting as adapters in conjunction with TNL proteins.

CONCLUSION

We have presented several lines of evidence consistent with some TN and TX proteins functioning in plant defenses. A small number of TN proteins are well conserved from gymnosperms to flowering plants, and a larger set of TIR-containing proteins are found in dicots. A subset of *TX* and *TN* genes were induced in SA-treated Arabidopsis tissues, and *TX* and *TN* overexpression in tobacco and Arabidopsis resulted in phenotypes that were consistent with a role in disease responses. The ability of overexpression of TX and TN proteins to induce cell death in tobacco was dependent on EDS1. Other published data suggests that more than one NB-LRR protein can function together in the recognition of pathogens (Eitas and Dangl, 2010). The presence of the TNL::TX or TN::TNL protein fusion pairs in Arabidopsis genome (Meyers et al., 2002) is consistent with a cofunctional role of TX or TN proteins together with TNL proteins. Thus, the TX and TN proteins could function together with TNL proteins to facilitate pathogen recognition or downstream signaling (Supplemental Fig. S6), and the XTNX may have other nondefense roles in monocots and other plants.

Though the specific functions of the majority of the TX and TN plant proteins remain unclear, our experimental evidence suggests the possibility for a role of TX and TN proteins in plant defense system. The *TX* and *TN* genes were diverse in their expression patterns, as observed with a variety of biotic stress treatments. Very few *TN* and *TX* genes were induced with the exogenous application of SA, suggesting that not all proteins might be involved similarly in a single pathway. The induction of two *TN* genes, *AtTN3* and *AtTN11*, by the application of both SA and JA indicates the possibility of their role in the SA-JA cross talk. Consistent with their expression patterns, the TX and TN proteins also show diversity in their localization patterns, as observed with fluorescence tagging the proteins at their C terminus. The diversity in their localization patterns in the plant cell and their observed in vitro Y2H interactions with elicitor proteins and components of NBS-LRR proteins suggest a functional diversity of these TX and TN proteins. The presence of the XTNX protein orthologs (AtTN21 and AtTN17) in other plant genomes at a high rate of conservation signifies that these proteins are not lost in evolution and might play an important role.

MATERIALS AND METHODS

Bioinformatics Analysis

The complete set of protein sequences for TX and TN proteins was obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org) based on published lists (Meyers et al., 2002). The conservation of the *TN* and *TX* genes and their encoded proteins from other plant genomes was assessed using Jalview from Phytozome (http://www.phytozome.net). Arabidopsis (*Arabidopsis thaliana*) TN proteins AtTN21 and AtTN17 were used as reference sequences for comparison to plant protein databases. In the resulting sequence set and alignment, all ambiguous positions were removed for each sequence pair. There were a total of 1,556 positions in the final data set. A bootstrap consensus tree was inferred from 1,000 replicates using the neighbor-joining method (Saitou and Nei, 1987). Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using a Poisson correction method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Expression analysis of *TX* and *TN* genes used the Genevestigator microarray data sets (https://www.genevestigator.com) and public SBS data sets for biotic and abiotic stress responses (http://mpss.udel.edu/at_sbs). The expression of the Arabidopsis *TX* and *TN* genes was also presented as the sum of abundance of their expression in each of the available libraries. Assessment of posttranslational modifications was performed using Expasy (http://expasy.org/tools).

Plant Growth Conditions

Arabidopsis plants (Col-0) were grown in controlled environmental growth chambers. Soil-grown plants were grown in the growth chambers at 21°C in 72% relative humidity under 16-h days. Tobacco (*Nicotiana benthamiana*) plants were grown in controlled environmental growth chambers with a controlled temperature of 24°C and under 16-h days. Tobacco plants were used at the 3-week stage for inoculations and *Agrobacterium tumefaciens* infiltrations.

RNA Isolation and cDNA Preparation

Plant RNA was isolated using TRIzol Reagent (Invitrogen), based on the manufacturer's recommendations. Following RNA isolation, complementary DNA (cDNA) was made from the total RNA using the SuperScript III RT kit for RT-PCR from Invitrogen according to the manufacturer's recommendations. RT was performed with SuperScript III reverse transcriptase followed by PCR amplification of the cDNA.

Real-Time PCR Analysis

Quantitative real-time PCR was carried out using 2X QuantiTect SYBR Green PCR Master Mix on an Applied Biosystems Fast System 7500 cycler. The PCR conditions were followed according to the manufacturer's recommendations (Qiagen). The PCR amplification protocol consisted of a 10-min denaturation step followed by 40 cycles of 15 s at 95°C, 30 s at 54°C, and 30 s at 72°C. The primers for the amplification of the TX and TN genes were used as listed (Supplemental Table S8).

SA and JA Treatments

SA solution of 100 μM was applied as foliar spray on Arabidopsis plants at the 3-week stage. One milliliter of 50 μM MeJA solution was applied as a volatile in a sealed 100-mL Magenta jar.

Plasmid Construction

The *TX* and *TN* genes were amplified from either cDNA or genomic DNA of Arabidopsis (Col-0). The primers for the amplification of the genes were designed manually based on the annotations in The Arabidopsis Information Resource database (Supplemental Table S9). Most cloning procedures utilized

the Gateway cloning technology (Invitrogen). The amplified PCR products were cloned into pDONR207 using the BP clonase (Invitrogen). The resulting sequences were then transferred into pK2GW7 or pEarleyGate 100 series vectors (pEG101, pEG102, and pEG103) using LR clonase (Invitrogen). The inserts were then cloned into plant binary vectors pCB302-rfB and pEarley-Gate 100 series vectors (used as a destination vectors), using the protocol from Invitrogen. The genes cloned in pK2GW7 and pEarleyGate 100 series expression vectors were driven by the 35S promoter. The nonredundant sets of clones in the final binary expression vectors pK2GW7 and pEarleyGate 100 series vectors were sequenced to confirm that they were identical to the corresponding clones in pDONR207.

Transient and Stable Transgenic Experiments

A. tumefaciens strain GV3101 was transformed with variants of pK2GW7 or pEarleyGate 100 series vectors using electroporation. Transient assays of tobacco were performed as described previously (Bernal et al., 2005). For all procedures, *A. tumefaciens* GV3101 containing pK2GW7 clones was grown in Luria-Bertani medium containing gentamycin at 50 μg mL^{-1} and rifampicin at 30 μg mL^{-1}. *A. tumefaciens* GV3101 containing pEarleyGate 100 series clones was grown in Luria-Bertani medium containing kanamycin at 50 μg mL^{-1} and rifampicin at 30 μg mL^{-1}. The resuspended *A. tumefaciens* cells were used to inoculate the leaves of tobacco on the abaxial surface of the leaves. Imaging under confocal microscope was done 42 h postinfiltration. Similar assays were performed in tobacco plants silenced for *EDS1* gene. *EDS1* in tobacco plants was silenced using VIGS, and AtTN3-dependent cell death was transiently induced in those plants as described previously (Swiderski et al., 2009).

The *A. tumefaciens*-mediated plant transformation of Arabidopsis was done using the floral-dip method (Clough and Bent, 1998). The T1 generation plants were screened on Murashige and Skoog plates with kanamycin. A minimum of six independent transgenics were tested per gene.

Pathogen Response Assays

For the fungal response assay, a root-dip inoculation method was followed. Ten-day-old seedlings of Arabidopsis wild-type Col-0 plants and the stable overexpression transgenic lines of *TX* and *TN* were grown in the soil. The mutant plants of *eds-1* and ecotypes Greenville and Cape Verde Island, ecotypes with known resistance to *Fusarium oxysporum*, were grown as controls. The roots were dipped in the fungal conidial suspension of concentration of 10^6 conidia mL^{-1} for one minute, and the seedlings were transferred to autoclaved soil. The plants were allowed to grow for another 3 weeks, and the plants were scored based on the number of plants dead or surviving.

For the bacterial response assay, Arabidopsis Col-0 plants and stable overexpression transgenic lines were grown until 3 weeks on soil pellets. Leaves of the plants were infiltrated with the virulent *Pseudomonas syringae* pv *tomato* DC3000 bacteria with an optical density at 600 nm value of 0.002 (approximately 1×10^6 colony forming units mL^{-1}). The leaves were left until dry on the surface, and the tray was covered with a dome until the completion of the experiment. Observations were recorded at 24, 48, and 72 h posttreatment. Bacterial growth analysis was performed as described previously (Melotto et al., 2006). Bacterial colonies were counted at each time point with at least three technical replicates.

HPLC Estimation of SA Levels

Leaf samples from Arabidopsis plants were weighed and then ground in liquid nitrogen; 400 μL of 80% (v/v) methanol was added, and samples were incubated overnight at 4°C with continuous agitation. The samples were centrifuged twice at 14,000 rpm, and the pellet was discarded. The supernatant was fractionated by HPLC with a 0% (v/v) to 100% (v/v) linear gradient of methanol in 2 mm formic acid, 30 μL total volume, and 60-min run time. Free SA levels in the plants were estimated using a C 18 column. The data were tabulated as microgram per gram of fresh weight.

Confocal Imaging

Live plant imaging was performed on a Zeiss LSM510 confocal microscope using a 40X C-Apochromat (NA = 1.2) water immersion objective lens. Tissue samples were cut from leaves of tobacco at approximately 42 h postinoculation (Caplan et al., 2008). The 458- and 514-nm laser lines of a 25-mW argon laser with appropriate emission filters were used to image cyan fluorescence and

yellow florescence, respectively. Membrane localization of the TN protein was confirmed using FM4-64 staining as an additional marker.

Y2H Experiments

Pairwise Y2H experiments were performed using the Matchmaker GAL4 Two-Hybrid System according to the manufacturer's recommendations (Clontech). TX and TN bait proteins were expressed as a fusion to the yeast transcription activator protein Gal4 (GAL4) DNA-binding domain, and libraries of prey proteins (pathogen elicitor proteins, plant signal transduction proteins, and NB-LRR proteins) were expressed as fusions to the GAL4 activation domain. The interactions were scored for their ability to grow on selective media lacking His and adenine for a double selection. Matings were replicated four times. The interactions were mapped using Cytoscape (Smoot et al., 2011).

The Y2H experiments on AtTN10 were performed by screening against cDNA prey libraries by Dualsystems using their DUALhybrid screening system. The cDNA library for target prey libraries was constructed using whole plant of Arabidopsis (equal mix of roots, leaves, and inflorescence) at 3-week stage. The complexity of the library was 2.7×10^7 independent clones with an average insert size of 1.8 kb.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogeny and conservation of TX and TN protein domains.

Supplemental Figure S2. Expression levels of *TN* and *TX* genes vary under biotic stresses.

Supplemental Figure S3. Western blots of the TN proteins AtTN10 and AtTN3 using anti-GFP antibodies confirm protein expression and localization in the nucleus and cytoplasm.

Supplemental Figure S4. Yeast two hybrid interactions of TX and TN proteins with candidate elicitor protein library.

Supplemental Figure S5. Colocalization of YFP with 3PGDH-CFP or AtTN10-YFP with CFP resulted in cytoplasmic localization.

Supplemental Figure S6. Arabidopsis 3PGDH protein At1g17745 is well conserved among other plant genomes.

Supplemental Figure S7. Model for the function of Arabidopsis TN and TX proteins.

Supplemental Table S1. Primers designed for the amplification of transcript abundance through the use of real time PCR.

Supplemental Table S2. Primers designed for the amplification of TX and TN transcripts for cloning and generation of pENTRY gateway vectors.

Supplemental Table S3. Expression data from rice orthologs of Arabidopsis *TIR-NBS* genes.

Supplemental Table S4. Arabidopsis TX and TN proteins used for HR assays on *N. benthamiana*.

Supplemental Table S5. Phylogeny and conservation of TN protein domains among monocots and other basal angiosperms.

Supplemental Table S6. Expression of *TN* and *TX* genes from public MPSS database libraries.

Supplemental Table S7. Stable transgenic overexpression lines generated in Arabidopsis ecotype Col-0.

Supplemental Table S8. Salk T-DNA knockout lines for *TX* and *TN* genes.

Supplemental Table S9. Summary of TX and TN phenotypes.

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