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Misexpression of *AtTX12* encoding a Toll/interleukin-1 receptor domain induces growth defects and expression of defense-related genes partially independently of EDS1 in *Arabidopsis*

*Sang-Kee Song**
Department of Biology, Chosun University, Gwangju 61452, Korea

In this study, a tissue-specific GAL4/UAS activation tagging system was used for the characterization of genes which could induce lethality when ubiquitously expressed. A dominant mutant exhibiting stunted growth was isolated and named *defective root development 1-D* (*drd1-D*). The T-DNA tag was located within the promoter region of *AtTX12*, which is predicted to encode a truncated nucleotide-binding leucine-rich repeat (NLR) protein, containing a Toll/interleukin-1 receptor (TIR) domain. The transcript levels of *AtTX12* and defense-related genes were elevated in *drd1-D*, and the misexpression of *AtTX12* recapitulated the *drd1-D* phenotypes. In the presence of ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), a key transducer of signals triggered by TIR-type NLRs, a low-level of *AtTX12* misexpression induced strong defective phenotypes including seedling lethality whereas, in the absence of EDS1, a high-level of *AtTX12* misexpression induced weak growth defects like dwarfism, suggesting that AtTX12 might function mainly in an EDS1-dependent and partially in an EDS1-independent manner. [BMB Reports 2016; 49(12): 693-698]

INTRODUCTION

To survive as sessile organisms, plants exhibit enormous plasticity in growth and development in response to various external cues, including biotic and abiotic stresses. Biotic stresses mainly originate from plant pathogens and herbivores, against which plants have evolved a wide range of defense mechanisms. Plants protect themselves from invasion by biotrophic or hemibiotrophic pathogens through innate immune responses. Resistance against non-specific pathogens is mediated by pattern recognition receptors which can detect conserved molecules such as bacterial flagellin and elongation factor Tu and lead to pathogen-associated molecular pattern-triggered immunity (PTI). However, host-specific pathogens have evolved the ability to deliver effector molecules into host cells, which allow them to evade PTI. Plants have co-evolved diverse resistance (R) proteins in response that sense these effectors or the host proteins that are modified by the effectors. R proteins induce stronger, pathogen-specific resistance responses, and are part of what is known as effector-triggered immunity (ETI), which restricts pathogen proliferation via local programmed cell death, also known as the hypersensitive response (1, 2).

Most R proteins are intracellular immune receptors with nucleotide binding (NB) and leucine-rich repeat (LRR) domains (NLR), possessing either a Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) domain at the N-terminus (3, 4). NLRs with TIR domains (TNLs) require ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), a eukaryotic lipase-like protein, and PHYTOALEXIN DEFICIENT 4 (PAD4), an EDS1-interacting partner, for downstream immune responses, including salicylic acid (SA)-mediated signaling, whereas most NLRs with a coiled-coil (CC) domain (CNLs) require NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) for immune signaling (5-8). TIR domains are reported to serve as platforms for homo- or hetero-dimerization to induce cell death signaling or effector perception (9-11). The NB domains of NLRs function as internal switches for conformational changes, depending on the status of the bound nucleotide. In the activated state, TIR/CC domains can be exposed to trigger defense signals. Overexpression of TIR regions of TNLs lacking NB domains induced constitutively active immune responses in the absence of effectors (11-14).

In *Arabidopsis* Col-0, there are 92 TNLs, 23 truncated TIR-NBs (TNs) lacking LRR domains, and 30 TIR-unknown domain (TX) proteins lacking both NB and LRR domains (15).

*Corresponding author. Tel: +82-62-230-6655; Fax: +82-62-230-6650; E-mail: sangkeesong@chosun.ac.kr

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The functions of a few *TX* genes have been characterized by stable/transient overexpression (16, 17). It is an intriguing question to answer whether or not the uncharacterized TXs possess activity for defense-related gene expression and follow typical TNL-mediated signaling to broaden our understanding of plant defense-related systems.

Activation tagging screening is an efficient way to characterize the unknown functions of redundant genes. Conventional activation tagging systems use viral enhancers to strongly and ubiquitously induce the expression of tagged genes (18), whereas the GAL4/UAS system (19) enhances flanking gene expression in a tissue-specific manner, which is advantageous for screening genes that induce lethality when ubiquitously expressed.

In this study, a UAS-tagged *defective root development 1-D* (*drd1-D*) mutant displaying stunted growth was isolated, and the tagged gene was identified as *AtTX12*, which encodes a truncated TNL lacking both the NB and LRR domains. Genetic analyses have shown that AtTX12-mediated growth defects and defense-related gene expression are independent of PAD4/NDR1 and partially independent of EDS1.

RESULTS

Isolation of the *drd1-D* and localization of the tagged gene

A dominant mutant exhibiting arrested growth named *defective root development 1-D* (*drd1-D*) was isolated by introducing UAS enhancers into the Q2610 enhancer trap line. The putative homozygous *drd1-D* was seedling lethal, displaying little post-embryonic development (Fig. 1A, C) compared to wild type (WT) (Fig. 1B, D) as the Q2610 is highly active in post-embryonic roots (19).

Thermal asymmetric interlaced (TAIL)-polymerase chain reaction (PCR) analysis revealed the DNA sequences between the T-DNA and genomic DNA, which are shown in Fig. 1E. The T-DNA, which contains UAS elements, was located 215 bp upstream of the putative start codon of *At2g03300*, previously designated as *AtTX12*, encoding a 203-amino acid (aa) truncated TNL containing a TIR domain and a 59-aa extension (17). The genome of the Col-0 contains *AtTX11* (*At2g03030*), which is the closest homologue of *AtTX12* probably originated from local gene duplication (15). The TIR domain of AtTX12 shares 34-54% aa-sequence identity with those of TNLs such as RPS4, RPP1, N, and L10, and contains 4 conserved TIR motifs (Supplementary Fig. 1) (15). AtTX12-like proteins are conserved among species of the Brassicaceae family, with 66-94% identity over the entire sequence (Supplementary Fig. 2).

Reverse transcription (RT)-PCR analysis showed the increased expression of *AtTX12* in *drd1-D* compared to that in WT (Fig. 1F). It was also examined whether the transcript levels of defense-related genes increased in *drd1-D* as *TX*-overexpressing plants are known to exhibit elevated defense-related responses (16, 17). The transcripts of defense-related genes such as *PATHOGENESIS-RELATED 1* (*PR1*), *β-1,3-GLUCANASE 2* (*BGL2*) (20), *EDS1*, and *PAD4* were highly accumulated in *drd-1D* compared to those in WT (Fig. 1F), suggesting that *AtTX12* may induce the expression of downstream defense-related genes.

As shown in Fig. 1G, endogenous *AtTX12* was expressed at a very low level in the roots and leaves, and tended to increase as the plants matured. To examine the subcellular localization of AtTX12, transgenic plants expressing *AtTX12pro:AtTX12-GFP* were screened based on the semi-

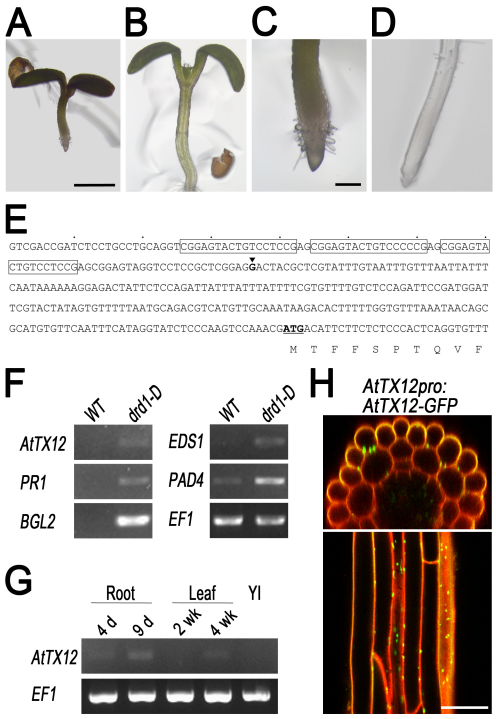

Fig. 1. The *drd1-D* exhibits stunted growth, which is induced by the misexpression of *AtTX12*. (A, B) The above-ground phenotypes of *drd1-D* (A) and WT (B). Scale bar = 1 mm. (C, D) The root tip phenotypes of *drd1-D* (C) and WT (D). Scale bar = 200 μm. (E) The T-DNA insertion site in *drd1-D* is located within the *AtTX12* promoter. The 3 UAS elements in the T-DNA insert and the 5' start point of the *AtTX12* promoter are shown in boxes and with an arrowhead, respectively. The putative start codon of *AtTX12* is shown in boldface and underlined. (F) RT-PCR analysis of defense-related gene expression in 4-d-old *drd1-D* seedlings. *EF1* was used as a control for the 25-cycle PCR. (G) RT-PCR analysis of organ-specific *AtTX12* expression in roots of 4 and 9-d-old seedlings grown in 0.5× MS media, 2- and 4-wk-old leaves, and young inflorescence (YI). *EF1* was used as a control for the 35-cycle PCR. (H) Subcellular localization of an AtTX12-GFP translational fusion protein expressed by a 1.8-kb *AtTX12* promoter. GFP signals were mainly detected in the plasma membrane and plasmodesmata connecting neighboring cells in the root as shown in the composite cross section (upper panel) and transverse section (lower panel) images. Scale bar = 40 μm.

dwarf to dwarf phenotypes. RT-PCR analysis showed that the expression of *AtTX12* was well-correlated with the phenotypic strength (Supplementary Fig. 3A, 3B). AtTX12-GFP signal was found in either the plasma membrane or plasmodesmata connecting neighboring cells in the root (Fig. 1H), suggesting that the AtTX12 might function in such subcellular regions.

Misexpression of *AtTX12* recapitulates the *drd-1D* phenotype

To further confirm that the *drd1-D* phenotype is induced by misexpression of *AtTX12*, transgenic plants harboring *AtTX12* under the regulation of the 5× UAS promoter (*UASpro:AtTX12*) were screened and crossed to Q2610 to generate a double hemizygous plant for Q2610 and *UASpro:AtTX12* (*Q2610/+>>AtTX12/+*). *Q2610/+>>AtTX12/+* displayed seedling lethality (Fig. 2A), phenocopying the homozygous *drd1-D*, indicating that the *drd1-D* phenotype originated from the misexpression of *AtTX12*. Two representative transgenic lines exhibited almost the same phenotypes displaying local cell death and pigment accumulation around the root tips (Fig. 2B, C), although *Q2610/+>>AtTX12/+* line #2 accumulated a little more *AtTX12* transcripts than line #1 (Fig. 2F). RT-PCR analysis showed increased expression of defense-related genes, such as *PR1*, *EDS1*, and *PAD4* in the *Q2610/+>>AtTX12/+* seedlings (Fig. 2F). These results suggest that the transcript level of *AtTX12* in *Q2610/+>>AtTX12/+* line #1 might be enough to induce seedling lethality.

To test whether the *AtTX12* misexpression phenotype is temperature-sensitive or not, *Q2610/+>>AtTX12/+* line #2 seedlings were grown at 28°C as TNL-mediated growth defects are often suppressed at a higher temperature (21). As shown in Fig. 2D, no phenotypic change of seedlings harboring transgenes was observed at a high-temperature condition.

To determine whether *AtTX11* induces growth defects like *AtTX12*, transgenic *UASpro:AtTX11* lines were prepared and crossed to Q2610. The *Q2610/+>>AtTX11/+* seedlings also exhibited seedling lethality (Fig. 2E).

To test whether tissue or organ-specific growth inhibition is induced by *AtTX12*, *AtTX12* was expressed by various enhancers. *J1721/+>>AtTX12/+* line #2 displayed root defects lacking a root tip as *J1721* is highly expressed in the columella root cap and protoxylem during embryogenesis and the phenotype was not recovered at 28°C (Fig. 2G, H). *Q0990/+>>AtTX12/+* line #2 exhibited early termination of primary root growth as *Q0990* is expressed in the stele initials at a low level, but even this relatively weak phenotype was not rescued at 28°C (Fig. 2I, J). *Q0990/+>>AtTX12/+* line #1 survived and produced double homozygous *Q0990>>AtTX12* line #1 (Fig. 2K). When *AtTX12* was expressed under the regulation of the *PISTILLATA* (*PI*) promoter, which is reported to be almost petal-specifically active (22), *PI/+>>AtTX12/+* line #1 displayed suppressed petal and stamen development, mimicking the *pi* phenotype, whereas the development of neighboring floral organs was almost unaffected (Fig. 2L, 2M) suggesting that *AtTX12* expression could induce tissue-specific local cell death.

Defense-related gene expression induced by *AtTX12* misexpression is mainly dependent on EDS1 and partially independent of EDS1

EDS1/PAD4 and NDR1 are generally required for TNL- and CNL-mediated defense-related responses, respectively. It was tested whether the AtTX12 activity is dependent on EDS1,

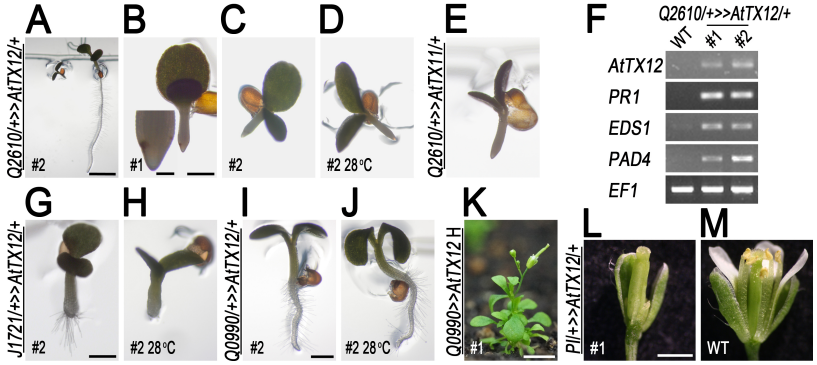

Fig. 2. Ectopic expression of *AtTX12/11* recapitulates the *drd1-D* phenotype by inducing defense-related gene expression. (A-D) Stunted growth phenotypes of double hemizygous *Q2610/+>>AtTX12/+* seedlings 4 d after germination. (A) A *Q2610/+>>AtTX12/+* (left) and WT-like sibling (right) of the F_1 progenies of *UASpro:AtTX12* line #2 crossed to Q2610. (B) A *Q2610/+>>AtTX12/+* line #1 seedling exhibiting local cell death and pigment accumulation. The inset shows an enlarged root tip. (C, D) *Q2610/+>>AtTX12/+* line #2 seedlings grown at 22°C (C) and at 28°C (D). (E) A 4-d-old *Q2610/+>>AtTX11/+* seedling. (F) RT-PCR analysis of *AtTX12*, *PR1*, *EDS1*, *PAD4*, and *EF1* expression in 4-d-old *Q2610/+>>AtTX12/+* line #1 and #2 seedlings in 25-cycle reactions. *EF1* was used as a control. (G, H) *J1721/+>>AtTX12/+* line #2 seedlings grown at 22°C (G) and 28°C (H). (I, J) *Q0990/+>>AtTX12/+* line #2 seedlings grown at 22°C (I) and 28°C (J). (K) The dwarf phenotype of a 5-wk-old double homozygous *Q0990>>AtTX12* line #1. (L, M) Defective petal and stamens of *PI/+>>AtTX12/+* line #1 (L) and a WT-like flower (M). A sepal was removed to expose the inner whorls. Scale bars = 5 mm (A), 0.5 mm (B-E), 0.1 mm (inset of panel B), 0.5 mm (G, H), 0.5 mm (I, J), 5 mm (K), and 1 mm (L, M).

PAD4, or NDR1. *Q2610/+>>AtTX12/+* line #2 *pad4-1* exhibited a seedling-lethal phenotype (Fig. 3A), suggesting that PAD4 may not be essential for AtTX12-triggered growth defects (Fig 3A). RT-PCR analysis showed that *AtTX12* misexpression led to increased transcript accumulation of defense-related genes in the *pad4-1* background (Fig. 3B). The seedling-lethal phenotype was consistently observed when *Q2610/+>>AtTX12/+* line #2 was introduced into 2 different putative *pad4* mutants, SALK_206216 and SALK_206548 (data not shown). *Q2610/+>>AtTX12/+* line #2 *ndr1-1* also exhibited a seedling-lethal phenotype. These results suggest that AtTX12 might function PAD4- and NDR1-independently (Fig. 3A).

As there is an additional isoform (*At3g48080*) of *EDS1* (*At3g48090*) in the genome of the Col-0, the T-DNA insertion mutants *eds1-22* (SALK_071051) and *eds1-23* (SALK_057149), which originated from Col-0, still possess partial EDS1 activity, whereas *eds1-2*, which originated from the L*er* possessing a single copy of *EDS1*, could be regarded as a null mutant. In this study, *eds1-2* mutant introgressed into Col-0 (23) was used for the genetic analyses. Seedlings of *Q2610/+>>AtTX12/+* line #2 in the *eds1-22*, *eds1-23*, and *eds1-2* backgrounds all displayed partially rescued, moderately short (approximately 65-70% of WT), weakly hairless, and twisted root phenotypes (Fig. 3A). However, at later developmental stages, *Q2610/+>>AtTX12/+* line #2 *eds1-22* and *eds1-23* exhibited dwarf, semi-sterile, and leaf chlorosis phenotypes, whereas *Q2610/+>>AtTX12/+* line #2 *eds1-2* did not exhibit any apparent growth defective phenotype (Fig. 3C). These results suggest that the *EDS1* isoform, *At3g48080* may be more active or abundant in above-ground organs than in the roots and better transduce signals triggered by AtTX12 in these organs.

Therefore, the plant homozygous for both Q2610 and *UASpro:AtTX12* line #2 (*Q2610>>AtTX12* line #2) was obtained only in the *eds1-2* mutant background at the F_4 generation by examining the presence of *Q2610*-GFP and the *UASpro:AtTX12*. *Q2610>>AtTX12* line #2 *eds1-2* displayed a shorter and more twisted root phenotype (Fig. 3A) compared to *Q2610/+>>AtTX12/+* line #2 *eds1-2*, suggesting that AtTX12 may function in an expression level-dependent manner. This tendency was also found in line #1, as the *Q2610/+>>AtTX12/+* line #1 *eds1-2* displayed little defect (data not shown), whereas homozygous *Q2610>>AtTX12* line #1 *eds1-2* displayed moderate root defects (Fig. 3A). These results suggest that when highly expressed, *AtTX12* may induce growth defects even in the absence of EDS1 through a putative EDS1-independent pathway.

RT-PCR analysis of *Q2610/+>>AtTX12/+* line #2 *eds1-22* and *eds1-23* showed increased expression of defense-related genes, such as *PR1* and *BGL2* possibly due to the presence of the *EDS1* isoform transducing the defense-related signals efficiently from the relatively small amount of *AtTX12* transcripts, whereas *Q2610/+>>AtTX12/+* line #2 *eds1-2* showed little defense-related gene expression due to the

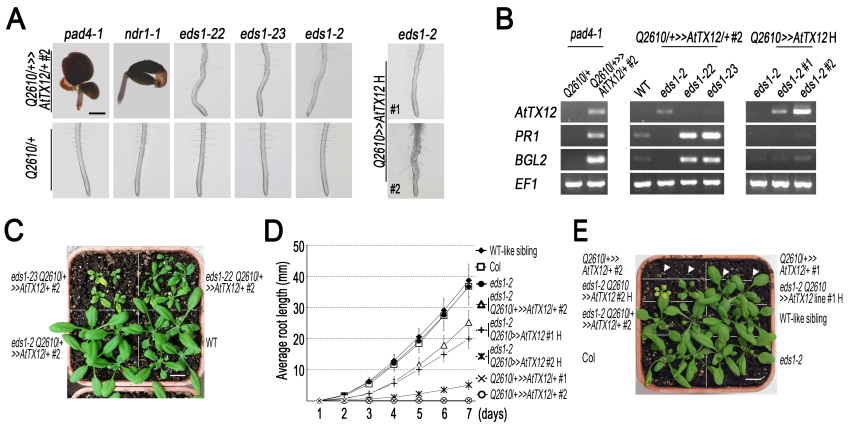

Fig. 3. The growth inhibition activity of *AtTX12* is independent of *PAD4/NDR1* and partially independent of *EDS1*. (A) Left, root tip phenotypes of 4-d-old seedlings of double hemizygous *Q2610/+>>AtTX12/+* line #2 (upper lanes) and *Q2610/+* controls (lower lanes) in various plant defense-related signaling mutants, including *pad4-1*, *ndr1-1*, *eds1-22*, *eds1-23*, and *eds1-2*.; Right, root tips of double homozygous *Q2610>>AtTX12* line #1 and #2 in *eds1-2*. Scale bar = 0.5 mm. (B) RT-PCR analysis of *AtTX12*, *PR1*, *BGL2*, and *EF1* expression in 4-d-old *Q2610/+>>AtTX12/+* line #2 *pad4-1* seedlings (left), 2-wk-old above-ground tissues of *Q2610/+>>AtTX12/+* line #2 *eds1-2*, *1-22*, and *1-23* with a WT control (middle) in a 25-cycle reaction, and 1-wk-old above-ground tissues of *Q2610>>AtTX12* line #1 and #2 in *eds1-2* in a 30-cycle reactions (right). (C) Above-ground phenotypes of *Q2610/+>>AtTX12/+* line #2 *eds1-2*, *eds1-22*, and *eds1-23* along with WT controls at 3 wks. after germination. Scale bar = 10 mm. (D) Average root length of *eds1-2* or WT seedlings that misexpressed transgenic *AtTX12* (*UASpro:AtTX12* line #1 or line #2), hemizygously or homozygously with Q2610, together with controls of Col, *eds1-2*, and WT-like siblings. The root length shown is an average of 8 seedlings. Error bars indicate SD. (E) Above-ground phenotypes of 3-wk-old plants described in panel (D). Scale bar = 10 mm. White lines were drawn to delineate plants of different genetic backgrounds (C and E).

absence of any functional *EDS1* thereby withstood the increased expression of *AtTX12* and remained healthy (Fig. 3B, 3C). The increase of the *PR1*/*BGL2* expression was detected in the *Q2610>>AtTX12* line #2 *eds1-2* at a relatively higher number of PCR cycles (Fig. 3B), suggesting that the *PR1*/*BGL2* expression induced by *AtTX12* misexpression may be mainly dependent on EDS1 and partially independent of EDS1.

AtTX12-mediated growth inhibition in *eds1-2* was well-correlated with the *AtTX12* transcript level, as displayed in the root growth curve. *Q2610>>AtTX12* line #2 *eds1-2* developed shorter roots compared with *Q2610/+>>AtTX12/+* line #2 *eds1-2* and *Q2610>>AtTX12* line #1 *eds1-2* (Fig. 3D, Supplementary Fig. 4). Furthermore, only the *Q2610>>AtTX12* line #2 *eds1-2* developed leaf chlorosis and a dwarf phenotype in the above-ground organs (Fig. 3E) which correlate well with increased *PR1*/*BGL2* gene expression (Fig. 3B). Together, these results further support the idea that AtTX12 can induce growth defects and defense-related gene expression partially EDS1-independently in a dose-dependent manner.

DISCUSSION

It is an intriguing question how plants defend themselves against a myriad of plant pathogens using a limited number of *R* genes. In addition to the canonical NLRs, there exist a number of truncated NLRs, such as TNs and TXs of which the roles in defense-related gene expression have not been well characterized with a few exceptions (16, 17, 24). In this study, it was shown that AtTX12, containing a TIR domain plus a short extension, could trigger defense-related gene expression possibly providing more diverse mechanisms for growth regulation and/or plant defense.

The AtTX12-GFP signal was mostly observed in either plasma membrane or plasmodesmata (Fig. 1H) although AtTX12 is predicted to possess a putative nuclear localization signal (NLS; KKKRKDCKCELPDLKKSRTKK) at the C-terminus (aa 182–202) (25). Therefore, it is needed to be tested whether this putative NLS is required for AtTX12 activity as reported in RPS4 (26).

Endogenous levels of *AtTX12* expression did not affect plant growth. However, when *AtTX12* expression exceeds a threshold level, the excessive AtTX12 is likely to induce growth defects and defense-related gene expression in an EDS1-dependent or -independent manner. In the presence of functional EDS1, low levels *AtTX12* misexpression can efficiently induce a defective growth phenotype together with defense-related gene expression (Fig. 2F), leading to a strong seedling-lethal phenotype (Fig. 2B). In contrast, in the absence of EDS1, only high levels of *AtTX12* misexpression can induce growth defects, albeit less efficiently with weakly increased defense-related gene expression (Fig. 3B), resulting in weaker defective phenotypes, such as dwarfism and leaf chlorosis (Fig. 3E). The growth defects induced by *AtTX12* in the absence of EDS1 might be transduced through PAD4/SENESCENCE ASSOCIATED GENE 101 (27) or, as yet unknown molecules present in the C24 ecotype, with lower efficiency compared to EDS1. As shown in Fig. 3A and C, misexpression of *AtTX12* in the *pad4-1* background induced growth defects together with defense-related gene expression, which is partially correlated with the previous observation that PAD4 is not required for the local acute response induced by TNLs but is required for basal immune responses (28).

Together, it is concluded that AtTX12 could induce growth defects and defense-related gene expression mainly in an EDS1-dependent and partially in an EDS1-independent way.

MATERIALS AND METHODS

Plant materials and growth conditions

GAL4/UAS enhancer trap lines, *pad4-1*, 2 *pad4* T-DNA insertion mutants (SALK_206548 and SALK_206216), *ndr1-1*, and *eds1-22* (SALK_071051) were obtained from the Arabidopsis Biological Resource Center (USA). Col *eds1-2* (23) and *eds1-23* (SALK_057149) were kindly provided by Tae-Houn Kim (Duksung Women's University, Korea). The Q2610 line used in this study was selected based on the non-late flowering phenotype after backcrossing to Col-0. Plants were grown as described in the supplementary materials.

Mutant screening and determination of the T-DNA insertion site

UAS-tagged mutants from the T_1 pool of Q2610 transformed with the pBIB-UAS vector kindly provided by Keiji Nakajima (NAIST, Japan) (19) were screened on 0.5× MS medium containing hygromycin B and then moved to normal medium for observation. TAIL-PCR was performed as described in the Supplementary Materials and Methods.

Preparation of gene constructs and genotyping markers

Gene constructs and genotyping markers were prepared as described in the Supplementary Materials and Methods.

Gene expression analyses

For the RT-PCR analysis, 1 μg of total RNA extracted from the various tissues of WT, mutant, and transgenic plants at various developmental stages was used for RT as described previously (29) and PCR amplified with gene-specific primers for *AtTX12* (5'-GACTAGTATGACATTCTTCTCTCCCACT-3' and 5'-TACTA GTTCACAACTTTTTGGTTCTGCTT-3') and other genes as described previously (16).

Confocal microscopy

The AtTX12-GFP signal was detected with a Zeiss LSM700 confocal microscope after seedlings were stained as described previously (29).

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