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Overexpression of three related root-cap outermost-cell-specific C2H2-type zinc-finger protein genes suppresses the growth of Arabidopsis in an EAR-motif-dependent manner

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The root meristem of *Arabidopsis thaliana* is protected by the root cap, the size of which is tightly regulated by the balance between the formative cell divisions and the dispersal of the outermost cells. We isolated an enhancer-tagged dominant mutant displaying the short and twisted root by the overexpression of *ZINC-FINGER OF ARABIDOPSIS THALIANA1* (*ZAT1*) encoding an EAR motif-containing zinc-finger protein. The growth inhibition by *ZAT1* was shared by *ZAT4* and *ZAT9*, the *ZAT1* homologues. The *ZAT1* promoter was specifically active in the outermost cells of the root cap, in which ZAT1-GFP was localized when expressed by the *ZAT1* promoter. The outermost cell-specific expression pattern of *ZAT1* was not altered in the *sombrero* (*smb*) or *smb bearskin1* (*brn1*) *brn2* accumulating additional root-cap layers. In contrast, ZAT4-GFP and ZAT9-GFP fusion proteins were distributed to the inner root-cap cells in addition to the outermost cells where *ZAT4* and *ZAT9* promoters were active. Overexpression of *ZAT1* induced the ectopic expression of *PUTATIVE ASPARTIC PROTEASE3* involved in the programmed cell death. The EAR motif was essential for the growth inhibition by *ZAT1*. These results suggest that the three related *ZAT*s might regulate the maturation of the outermost cells of the root cap. [BMB Reports 2020; 53(3): 160-165]

INTRODUCTION

Plants as sessile organisms rely on their root systems to anchor themselves in the soil and to deliver water and nutrients to aerial organs. The primary root of Arabidopsis (*Arabidopsis thaliana*) consists of discrete concentric tissues, such as the root cap, epidermis, cortex, endodermis, and stele, from outside to inside. These tissues arise continuously from the specially organized group of cells named the root apical meristem (RAM) residing at the root tip. The quiescent center (QC) composed of around four slowly dividing cells resides at the very center of RAM and is supposed to emit signals suppressing the differentiation of the surrounding stem cells, such as the columella, epidermis/lateral root cap (EPI/LRC), cortex/endodermis, and stele initials (1, 2).

The root caps of plants are required for the protection of the RAM, the perception of the environmental cues, and the interaction with the rhizosphere. Root caps originate in two separate ways, one from the columella initial and the other from the EPI/LRC initials, which provide LRC and epidermis by periclinal division and anticlinal division, respectively (3). *WUS RELATED HOMEBOX 5* (*WOX5*) is essential for the maintenance of columella stem cells (CSCs), as shown by the loss-of-function and the gain-of-function studies (4). WOX5 is mobile and suppresses the expression of *CYCLING DOF FACTOR 4* which in turn increases the root-cap differentiation (5). *CLV3/ENDOSPERM SURROUNDING REGION* (*CLE*) 40 encoding a polypeptide hormone increases the differentiation of CSC, because *cle40* develops an additional layer of CSCs (6). The treatment CLE40 peptide induces the consumption of CSCs, whereas the loss-of-function in *ARABIDOPSIS CRINKLY4* and *CLAVATA1* suppresses this effect of CLE40 (7).

Several related NAM ATAF and CUC (NAC) domain transcription factors are required for the maintenance of the proper number of root-cap cell layers. *FEZ* promotes the formative cell divisions in the columella and the EPI/LRC initials, whereas *SOMBRERO* (*SMB*) increases the differentiation of the root cap. *FEZ* positively regulates *SMB* expression in the differentiating daughter cells, whereas *SMB* negatively regulates the *FEZ* expression, preventing additional formative cell divisions (8). In addition, *BEARSKIN1* (*BRN1*) and *BRN2* are involved in the cell sloughing-off in the root cap by regulating the expression of cell-wall degradation enzyme genes, such as the *CELLULASE5* (9) and *ROOT CAP POLYGALACTURONASE* (RCPG) genes (10, 11).

Recently, it was reported that INFLORESCENCE DEFICIENT IN

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ABSCISSION-LIKE1, a polypeptide hormone, and HAESA-LIKE2, a receptor kinase, signaling is required for the proper detachment of root-cap cells, possibly by regulating the expression of *RCPG* and *BIFUNCTIONAL NUCLEASE1* (*BFN1*), a gene related to developmental programmed cell death (dPCD) in the root cap (12). For the matured root-cap cell layers to be detached, the dPCD occurs at the LRC following the expression of *BFN1* and *PUTATIVE ASPARTIC PROTEASE3* (*PASPA3*) encoding an aspartic protease under the regulation of *SMB* and then triggers the detachment of the columella cells (13).

C2H2-type (C2H2) zinc-finger protein genes are involved in transcription regulation for the responses to abiotic stresses and developmental regulations (14). Some zinc-finger protein contains a transcriptional repressor domain known as the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs that regulate the gene expression negatively by the interaction with corepressor TOPLESS (TPL) and TPL-related proteins (TPRs) recruiting the histone deacetylase complex (15-17). Among the three-fingered C2H2 zinc-finger proteins, the function of *DUO1-ACTIVATED ZINC FINGER1* (*DAZ1*) and *DAZ2* are best characterized. They are required for the male germ cell divisions and can interact with corepressor TPL/TPR for the transcriptional suppression (18). However, the roles of other three-fingered zinc-finger protein genes have been poorly characterized (19, 20).

To screen for genes regulating the root development of Arabidopsis, we introduced 5x UAS activation tags into a Q2610 enhancer trap line, of which the root tip expresses GAL4-VP16 transcription factor abundantly (21), and then isolated a dominant mutant displaying a short and twisted root overexpressing a zinc-finger protein gene. Here, we report the functions and expression patterns of the three closely related three-fingered C2H2 zinc-finger protein genes (14).

RESULTS AND DISCUSSION

Isolation and characterization of *drd2-D* displaying short and twisted root phenotypes

We isolated a dominant mutant developing short and twisted roots named *defective root development 2-D* (*drd2-D*) by introducing the 5x UAS tag into a Q2610 enhancer trap line (Fig. 1B). The *drd2-D* displayed the compromised root epidermal patterning as shown by the non-hair cell markers (Supplementary Fig. S1). By thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) analysis, we found the insertion site of the UAS tag at the 132 base pairs (bps) upstream of the start codon of a C2H2-type zinc-finger protein gene, *ZINC-FINGER OF ARABIDOPSIS THALIANA* (*ZAT1*, At1g02030) (Fig. 1C) that was only partially characterized previously (22). The transcripts of *ZAT1* increased in *drd2-D* compared with the WT control, as shown by the reverse transcription (RT)-PCR analysis (Fig. 1D), and the transgenic expression of *UASp:ZAT1* under the control of Q2610 (*Q2610>>ZAT1*) induced severe growth inhibition in the root of transgenic seedlings

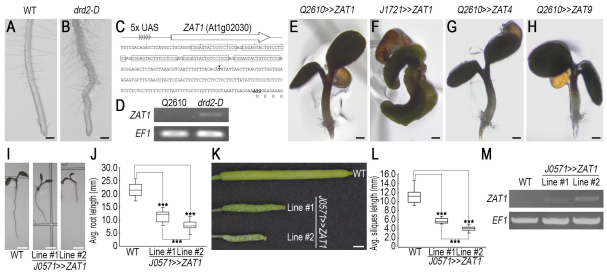

Fig. 1. Overexpression of *ZAT1* inhibits growth of various organs in transgenic plants including *drd2-D* mutant. (A, B) A *drd2-D* seedling exhibiting the short and twisted root phenotypes as compared with WT. (C) The T-DNA insertion site inducing *drd2-D* phenotype identified by the TAIL-PCR analysis. T-DNA is located at 144 bp upstream from the start codon of *ZAT1* (At1g02030). (D) An RT-PCR analysis showing the increased transcripts of *ZAT1* gene in the *drd2-D*. (E-H) The root-defective phenotypes of seedlings of *Q2610>>ZAT1* (E), *J1721>>ZAT1* (F), *Q2610>>ZAT4* (G), and *Q2610>>ZAT9* (H). (I-M) *J0571>>ZAT1* exhibits reduced root growth (I, J) and siliques length (K, L), which correlated with the *ZAT1* expression in transgenic lines identified by RT-PCR analysis (M). Bars = 200 μm in (A-I) and 1 mm in (K). ***P < 0.01, one-way-ANOVA (Tukey HSD), root n = 16; silique n = 13.

(Fig. 1E). When *UASp:ZAT1* was expressed during the embryogenesis by a *J1721* enhancer mimicking the expression of *MONOPTEROS* (*MP*) (23-25), the transgenic seedlings exhibited rudimentary roots reminiscent of the *mp* phenotype (Fig. 1F). The organization of RAM of the *Q2610>>ZAT1* seedling was compromised, as shown by the diffusively extended expression pattern of *WOX5p:GUS*, a QC marker (Supplementary Fig. S2A, B). In addition, the cell division was suppressed in the RAM of *Q0990>>ZAT1*, displaying defective growth and a reduced meristematic zone (Supplementary Fig. S2C, D), as shown by the reduced expression of *CYCB1;1p:GUS* (Supplementary Fig. S2E, F). These results indicate that *ZAT1* can inhibit the growth of Arabidopsis, leading to the complete loss of specific organs where it is highly expressed.

In the Arabidopsis genome, there are two *ZAT1*-related genes, *ZAT4* and *ZAT9*. ZAT1 possesses 43.4% and 43.8% amino-acid (a.a.) sequence identity with ZAT4 and ZAT9, respectively, and ZAT4 and ZAT9 exhibit 60.4% identity with each other (Supplementary Fig. S3). They share a conserved EAR motif at the C-terminal ends together with three conserved C2H2 zinc-finger motifs. Both *ZAT4* and *ZAT9* likely shared the function of *ZAT1* as *Q2610>>ZAT4* and *Q2610>>ZAT9* exhibited severe growth inhibition in the primary root (Fig. 1G, H) comparable to *Q2610>>ZAT1* (Fig. 1E). These results suggest that the three related *ZAT*s might function redundantly to suppress the growth of Arabidopsis in the specific tissues.

To find out whether *ZAT1* can affect the growth of aerial organs as well, we compared the organ size and the *ZAT1* transcription level in a weak transgenic line, *J0571>>ZAT1*, that can complete the whole life cycle by developing reproductive organs. The growth inhibition in the roots (Fig. 1I, J)

and siliques (Fig. 1K, L) was well correlated with the transcription level of *ZAT1* (Fig. 1M).

The outmost cell-specific expression patterns of *ZAT1, ZAT4,* and *ZAT9*

To examine the overall expression patterns of the *ZAT*s in various organs, we did RT-PCR analysis with gene-specific primers. The transcripts of *ZAT*s accumulated in the various tissues at the various developmental stages (Supplementary Fig. S4). To visualize the tissue-specific expression patterns of *ZAT*s, a *β-glucuronidase* (*GUS*) reporter was driven by each *ZAT* promoter in the transgenic plants. The three *ZAT*s commonly expressed in the outermost layer of the root cap (Fig. 2A, H, O). To identify the earliest developmental stage when *ZAT*s begin to express, we examined the GUS activity in the transgenic embryos. *ZAT*s did not express at the torpedo (Fig 2B, I, P) and bent cotyledon (Fig 2C, J, Q) stages when the three discrete root-cap layers are present.

The *ZAT1p:GUS* and *ZAT4p:GUS* began to express around 24 h after germination (Fig. 2D, K), whereas *ZAT9p:GUS* did around 36 h after germination (Fig. 2S). In addition to the root cap, the *ZAT1*-specific GUS expression was observed in the stele and endodermis in the root (Supplementary Fig. S5A-B) and hydathode (Fig. 2F). The *ZAT4*-specific expression was found in the guard cells (Fig. 2M) and pollen grains (Fig 2N), where the *ZAT9p:GUS* was active as well (Fig. 2U). These results indicate that the promoters of *ZAT*s are inactive during the embryogenesis and then are activated post-embryonically after the root tips are exposed to growth media.

The expression pattern of *ZAT1p:GUS* was not apparently altered in *drd2-D*, where *ZAT1* is highly expressed (Supplementary Fig. S5C) and in the *Q2610>>SMB* line, in which the root-cap layers increased (Supplementary Fig. S5D). In contrast, the number of root-cap layers expressing *ZAT1p:GUS* increased in *tyrosylprotein sulfotransferase* (*tpst*)*-1* developing an array of partially detached root-cap layers attached at the columella cells (26) (Supplementary Fig. S5E, F). These results suggest that *ZAT1* expression is not under the regulation of *ZAT1* and is activated when the root-cap cells are exposed to growth media. It was reported that the expression of *ZAT1* is responsive to nitrate (20, 27); however, this interaction might be indirect, because nitrate affects the development of lateral roots and thereby the '*ZAT1*-expressing' root caps as well (28).

The outermost layer-specific expression of *ZAT1* is not altered in the background-accumulating additional root-cap layers

To more precisely examine the *ZAT1*-expressing cell layers in the root cap, the *GFP5-ER* reporter and *ZAT1-GFP* were driven by the *ZAT1* promoter in the transgenic plants. Both *ZAT1p:GFP5-ER* and *ZAT1p:ZAT1-GFP* expression was specifically localized in the outermost cell layer of the root caps of wild-type seedlings (Fig. 3A, B, H-K). This layer-specific expression was not altered even in the background of *smb-3* and *smb-3 brn1-1 brn2-1* triple mutant, where additional root-cap layers

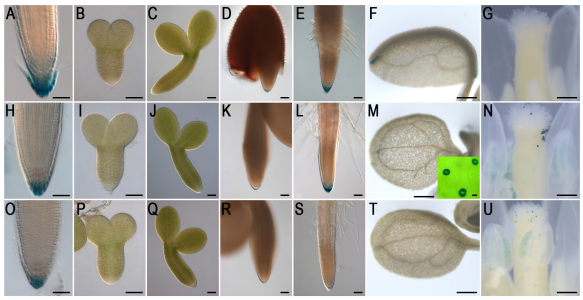

Fig. 2. Expression patterns of *ZAT1p:GUS*, *ZAT4p:GUS*, and *ZAT9p:GUS* reporter genes. *ZAT1p:GUS* (A-G), *ZAT4p:GUS* (H-N), and *ZAT9p:GUS* (O-U) expression in the root tips (A, H, O), torpedo-stage embryos (B, I, P), bent-cotyledon stage embryos (C, J, Q), seedlings 24 h after germination (D, K, R), 36 h after germination (E, L, S), cotyledons 5 d after germination (F), and open flowers (G, N, U). Inset in M is an enlarged view of the cotyledon. Bars = 50 μm in (A-E, H-L, O-S, inset in M), 200 μm in (F-G, M-N, T-U).

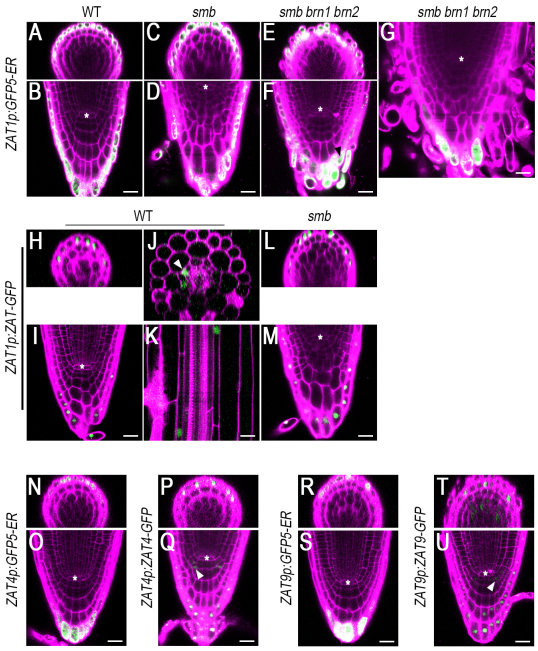

Fig. 3. The expression patterns of GFP5-ER and ZAT1/4/9-GFP fusion proteins driven by the respective *ZAT1/4/9* promoters in the transgenic roots. (A-G) *ZAT1p:GFP5-ER* expression in WT (A, B), *smb-3* (C, D), and *smb-3 brn1-1 brn2-1* (E-G). A black closed arrowhead in (F) indicates a newly exposed outermost cell. (H-M) *ZAT1p:ZAT1-GFP* expression in WT (H-K) and *smb-3* (L-M). (N-U) Reporter gene expression in transgenic roots expressing *ZAT4p:GFP5-ER* (N, O), *ZAT4p:ZAT4-GFP* (P, Q), *ZAT9p:GFP5-ER* (R, S), and *ZAT9p:ZAT9-GFP* (T, U). White arrowheads indicate nuclei expressing ZAT1-GFP (J), ZAT4-GFP (Q), and ZAT9-GFP (T). Asterisks indicate QCs. Bars = 50 μm.

accumulated (Fig. 3C-G, 3L, M). Interestingly, the underlying cells began to express the *ZAT1* as they were exposed to growth media, even though the ex-outermost cells was not completely detached (Fig. 3F). In addition to the root cap, ZAT1-GFP was localized in the nuclei of the endodermal cells (Fig. 3J, K). These results suggest that *ZAT1* promoter might not be gradually activated as the root-cap cells move away from the initial position, but are rather abruptly activated by the exposure to the growth media. Furthermore, *ZAT1* expression is likely regulated independently of *SMB*, *BRN1* and *BRN2*.

Both *ZAT4p:GFP5-ER* and *ZAT9p:GFP5-ER* were specifically expressed in the outermost layers of the root cap as well (Fig. 3N-O, 3R-S). Unlike ZAT1-GFP, ZAT4-GFP and ZAT9-GFP were broadly distributed to the inner root-cap cells even close to the CSCs (Fig. 3P, Q, 3T, U) together with the outermost cells. These results suggest that ZAT4 and ZAT9 proteins or *ZAT4* and *ZAT9* transcripts might be mobile, unlike ZAT1 or *ZAT1*. The discrepancy between the expression sites and the location of ZAT4-GFP and ZAT9-GFP fusion proteins implies that these proteins could transfer the growth inhibition signals from the outermost cells that can directly sense the environmental cues.

The expression of *PASPA3* was induced by the ectopic expression of *ZAT1*

To examine whether *ZAT1* regulates the expression of the root-cap-specific genes such as *SMB, CEL5*, and *PASPA3*, each GUS reporter driven by the respective promoter was introduced into the *Q2610>>ZAT1* background. The expression of *SMBp:GUS* and *CEL5p:GUS* was maintained at the root tip but reduced, probably because of the growth inhibition by *ZAT1* (Fig. 4A, B, 4G, H). In contrast, the expression of *PASPA3p:GUS* in the *Q2610>>ZAT1* seedlings was increased in the root-hypocotyl transition zone, where Q2610 is actively expressed (Fig. 4C-F, 4I-L). These results suggest that *SMB* and *CEL5* are not under the regulation of *ZAT1*, whereas *ZAT1* might be involved in the regulation of the *PASPA3* expression triggering dPCD.

The growth inhibition by *ZAT1* is dependent on the EAR motif

EAR motif is known to mediate the interaction between transcription repressors and TPL/TPR co-repressors to recruit histone deacetylase complexes (15). ZAT1 possesses two conserved EAR motifs, motif 1 (LSLML) present in the 130-134th a.a. and motif 2 (DLNLPA) in the 250-255th a.a. (16). Because motif 2 is well conserved among ZAT1, ZAT4, and ZAT9 (Supplementary Fig. S3), we introduced the single or double mutation into motif 2 by site-directed mutagenesis to investigate whether the EAR motif 2 is required for the growth inhibition (Fig. 4M).

Overexpression of the *UASp:ZAT1-L251A* or *UASp:ZAT1-L253A* single mutant driven by Q2610 induced the growth inhibition of transgenic seedlings comparable to that of *Q2610>>ZAT1* (Fig. 4N-P). However, the overexpression of *UASp:ZAT1-mEAR2* (L251; 253A) failed to inhibit the growth when we examined 30 independent transgenic lines (Fig. 4Q). These results indicate that the conserved EAR motif 2 is required for the growth inhibition by ZAT1.

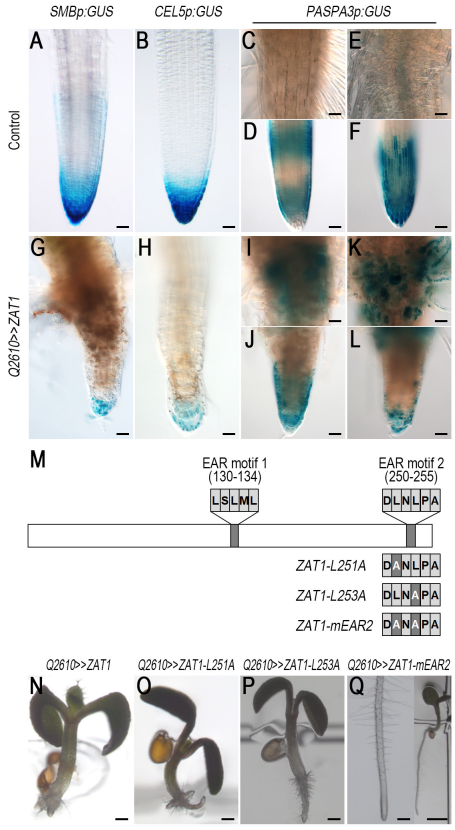

Fig. 4. The ectopic expression of *ZAT1* induced the mis-expression of *PASPA3* and the requirement of the EAR motif for the *ZAT1* activity for growth inhibition. (A-L) The expression patterns of root-cap reporters in the *Q2610>>ZAT1* background (G-L) as compared with control (A-F). *SMBp:GUS* (A, G), *CEL5p:GUS* (B, H), *PASPA3p:GUS* (C-F, I-L). Bars = 50 μm. (M) A diagram indicating the amino-acid sequences of EAR motif 2 of WT and EAR motif mutants of ZAT1. (N-Q) The phenotypes of 4-d-old transgenic seedlings of *Q2610>>ZAT1* (N), *Q2610>>ZAT1-L251A* (O), *Q2610>>ZAT1-L253A* (P), and *Q2610>>ZAT1-mEAR2* (Q). Inset in Q is a reduced view of the seedling. Bars = 200 μm in (N-Q) and = 1 mm in inset.

It was reported that transgenic plants overexpressing *ZAT7*, a C2H2 zinc-finger protein gene with an EAR motif, displayed salinity resistance together with growth-inhibition phenotypes. The EAR motif of ZAT7 was essential for the salinity resistance but not for the growth inhibition by ZAT7 (29). In contrast, the growth inhibition by ZAT1 in this study is dependent on the EAR motif of ZAT1, suggesting that the function of an EAR-motif differs for each zinc-finger protein.

Considering the growth inhibition activities, the roles of ZAT1 in the outmost cells might be to prevent them from further growth and to induce cell maturation after the exposure to the

media. Indeed, the *ZAT1* overexpression induced the ectopic expression of *PASPA3*, a marker for dPCD (30), in the hypocotyl-root transition zone, although it is not clear whether this interaction is direct or not. Because the endogenous expression patterns of *ZAT1* and *PASPA3* partially overlap in the columella and bottom of LRC, ZAT1 might regulate the *PASPA* expression in the corresponding tissues (13).

The shared functions of *ZAT*s might not be essential for normal growth, because a *zat1 zat4 zat9* triple mutant did not display any discernible phenotypes (Supplementary Fig. S6). However, in a specific environmental condition that we have not yet investigated, *ZAT*s might be required for the proper growth of Arabidopsis.

MATERIALS AND METHODS

Plant materials and growth condition

The T-DNA insertion site of *drd2-D* was identified as described previously (31). The T-DNA insertion lines SAIL_142_F08 (*zat1-1*), Wisceq_DsLox477-480D18 (*zat9-1*), *smb-3* (SALK_143526) (8), *brn1-1* (SALK_151986), and *brn2-1* (SALK_151604) (10) were obtained from Arabidopsis Biological Resource Center (ABRC), and GABIseq_181A01 (*zat4-1*) was obtained from Nottingham Arabidopsis Stock Centre. *GL2p:GUS* (32), *CYCB1;1p:GUS* (33), *WOX5p:GUS* (4), and *CPCp:GUS* (34) were described previously (35, 36). Plants were grown as described in the supplementary materials.

Gene expression analyses

GUS activity was defined histochemically by staining four-day-old seedlings as described (37). To investigate *ZAT*s transcripts expression level, we extracted total RNA from various tissues and used it for the templates for cDNA synthesis. We used *ELONGATION FACTOR1* (*EF1*) to normalize the relative levels of each transcript. Primers used in this study are given in Supplementary Table 1.

Confocal Microscopy

To detect GFP signals, we stained seedlings with 5 μg/mL propidium iodide (PI) and used a Zeiss LSM 880 confocal microscope (38). The GFP signals were detected with excitation at 488 nm and detected with a 493 to 544 nm band-path filter, PI signal was detected with a 604 to 718 nm long-path filter.

Preparation of gene constructs

Gene constructs were prepared as described in the supplementary materials and methods.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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