TITLE

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

AUTHORS

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RUNNING TITLE

ZAT1 with EAR-motif inhibits the growth of plants.

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SUPPLEMENTARY MATERIALS AND METHODS

Plant growth conditions

In order to grow seedlings in the sterile condition, seeds were sterilized using diluted bleach solution and were sown on 0.5x Murashige and Skoog (MS) medium solidified with gelite (Duchefa). Plates were put in a chamber at 4°C in the dark condition for 3 days for stratification. The seedlings were grown vertically for 4-5 days in the continuous light condition. After phenotypic observation of the seedlings, they were transferred to soil (Sunshine Mix #5, Sungro) containing 1 g of fertilizer per pot (Osmocote 14-14-14).

Cloning and transformation

To prepare the constructs for the overexpression of ZAT1, ZAT4, and ZAT9, genomic DNA fragments of ZAT1, ZAT4, and ZAT9 coding region were PCR amplified with primers ZAT1-F/ZAT1-R, ZAT4-F/ZAT4-R, and ZAT9-F/ZAT9-R, respectively. The PCR products were cloned into pJET2.1 cloning vector, the sequences were verified, and then each DNA fragment was inserted into pCB302 vector (Xiang et al. 1999) containing 5x UAS promoter and the nos terminator (Song et al. 2008). For the dPCD marker construct, about 2 kb promoter region of PASPA3 was PCR amplified with primers PASPA3pro-F and PASPA3pro-R by using the genomic DNA as a template. The fragment was cloned into pJET2.1 vector and its sequence was verified. The promoter fragment digested with SpeI/BamHI and then cloned into the binary vector pCB308 vector containing uidA gene. In a similar way, SMB, CEL5, ZAT1, ZAT4, and ZAT9 promoters were PCR amplified, cloned into a vector, sequence verified, digested with proper restriction enzymes and then cloned into pCB308 vector containing uidA gene (Xiang et al. 1999).

To prepare the ZAT1p:GFP5-ER and ZAT1p:ZAT1-GFP construct ZAT1 promoter was PCR amplified with ZAT1pro-F/ZAT1pro-R and inserted into the pCB302 vector (Xiang et al. 1999) containing nos terminator (ZAT1pro-nos-pCB302). And then GFP5-ER fragment or the ZAT1
fragment (amplified with ZAT1cod-F and ZAT1cod-R) fused to smRsGFP construct was inserted into the ZAT1pro-nos-pCB302 vector. In a similar way, ZAT4 and ZAT9 constructs were prepared. Plant transformation was performed using the floral dip method as previously described (Clough and Bent 1998). Information of primers used for cloning are given in the Supplemental table S1.

**Tissue-specific misexpression of ZATs**

For the tissue-specific misexpression of ZAT1 (or ZAT4, or ZAT9), a transgenic plant harboring UASp:ZAT1 (or ZAT4, or ZAT9) was crossed to a Haseloff’s enhancer trap line containing GAL4-VP16 (Haseloff 1998) for the transactivation (as indicated as ‘>>’) of UAS promoter by the GAL4-VP16 transcription factor. The expression patterns of enhancer trap lines such as J0571, J1721, Q0990 and Q2610 in the root tip were visualized previously (Song et al. 2015). The above-ground expression patterns of J0571 and Q2610 were visualized previously (Waki et al. 2013).
Supplemental Table 1.

| Target gene      | Primer   | Sequence                                      |
|------------------|----------|-----------------------------------------------|
| PASPA3 promoter  | Forward  | 5’-ACTAGTTTAGGGTTAGAATGAAAGAAAG3’             |
|                  | Reverse  | 5’-GGATCCTTTTACCTGTCATCAAAAAACAC3’           |
| SMB promoter     | Forward  | 5’-ACTAGTTGCAAGAACCCTAAGAAGCTCT3’            |
|                  | Reverse  | 5’-GGATCCTATCTCTTACTCTTTTAAGC3’              |
| CEL5 promoter    | Forward  | 5’-ACTAGTTGCAAGAACCCTAAGAAGCTCT3’            |
|                  | Reverse  | 5’-GGATCCTTTTACCTGTCATCAAAAAACAC3’           |
| ZAT1             | Forward  | 5’-AGGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT1 promoter    | Forward  | 5’-AGGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT1 coding      | Forward  | 5’-AGGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT4             | Forward  | 5’-GGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT4 promoter    | Forward  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
|                  | Reverse  | 5’-GGATCCATGGAAAGAGGGCATAAGTG3’             |
| ZAT4 coding      | Forward  | 5’-GGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT9             | Forward  | 5’-GGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT9 promoter    | Forward  | 5’-GGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| ZAT9 coding      | Forward  | 5’-GGATCCATGGAAAGAGGGCATAAGTG3’             |
|                  | Reverse  | 5’-CTCTAGATTTAAAAACCGAGGAGCCATCTTC3’         |
| smRsGFP          | Forward  | 5’-ACTAGTATGAGTTAAAAAGGAAGAAGCCTTTT3’        |
|                  | Reverse  | 5’-CTCTAGGTTATTTGATGATCCTGCTAC3’             |
| GFP5-ER          | Forward  | 5’-GGATCCATGGAAAGACTAAATCTTTTCTCT3’          |
|                  | Reverse  | 5’-CTCTAGATTTAGTTGCGTCGATCTTGTGTTGAT3’       |
| EF1              | Forward  | 5’-GGATCCATGGAAAGACTAAATCTTTTCTCT3’          |
|                  | Reverse  | 5’-CTCTAGGTTATTTGATGATCCTGCTAC3’             |
| pBIBLB372        | Forward  | 5’-GCAGCTGCGAGCAGAGGCTTTT3’                   |
| pBIBLB262        | Forward  | 5’-GCAGCTGCGAGCAGAGGCTTTT3’                   |
| pBIBL172  | 5’-GTCGACAGATCTCATGCCTGCA-3’ |
|-----------|-------------------------------|
| DEG1      | 5’-WGCNAGTNAGWANAAG-3’       |
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The plant journal 16:735-743

Haseloff J (1998) GFP variants for multispectral imaging of living cells. In: Methods in cell biology, vol 58. Elsevier, pp 139-151

Song S-K, Hofhuis H, Lee MM, Clark SE (2008) Key divisions in the early Arabidopsis embryo require POL and PLL1 phosphatases to establish the root stem cell organizer and vascular axis. Developmental cell 15:98-109

Song S-K, Kwak S-H, Chang SC, Schiefelbein J, Lee MM (2015) WEREWOLF and ENHANCER of GLABRA3 are interdependent regulators of the spatial expression pattern of GLABRA2 in Arabidopsis. Biochemical and biophysical research communications 467:94-100

Waki T, Miyashima S, Nakanishi M, Ikeda Y, Hashimoto T, Nakajima K (2013) A GAL 4-based targeted activation tagging system in Arabidopsis thaliana. The Plant Journal 73:357-367

Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant molecular biology 40:711-717
Supplementary Fig. S1. *drd2-D* exhibited the compromised root epidermal patterning.

The expression patterns of *GL2p:GUS* (A-B) and *CPCp:GUS* (C-D) in the *drd2-D* (B,D) were compromised as compared with those WT (A,C). Bars = 50 μm.
Supplementary Fig. S2. The compromised organization of the root meristem of Q2610>>ZAT1 seedling and the reduced cell divisions in the root meristem of Q0990>>ZAT1 seedling.

(A) WT and (B) Q2610>>ZAT1 harboring WOX5p:GUS. (C) WT and (D) Q0990>>ZAT1 seedling. (E) WT and (F) Q0990>>ZAT1 harboring CYCB1;1pGUS. The expression pattern of WOX5p:GUS was compromised in the root meristem of Q2610>>ZAT1 seedling (B) as compared with that in WT (A). The CYCB1;1pGUS expression in Q0990>>ZAT1 (F) was reduced as compared with that in WT (E) reflecting the reduced meristematic region of Q0990>>ZAT1 (D) as compared with that in WT (C). Arrowhead indicate compromised QC weakly expressing WOX5p:GUS. Bars = 50 μm (A-B, E-F), 200 μm (C, D).
Supplementary Fig. S3. Multiple amino acid sequence alignment among the three zinc-fingered C2H2 zinc finger proteins, ZAT1, ZAT2, and ZAT3.

The predicted EAR motifs are indicated in the square boxes and the conserved C2H2 zinc-finger motifs are shown in boldface.
Supplementary Fig. S4. The transcription levels of ZAT1, ZAT4, and ZAT9 expressed in various organs of Arabidopsis.

RT-PCR was performed with the cDNA synthesized by using total RNA isolated from various organs of Col-0 plants such as roots of 5, 7, 9, 11 d-old seedlings, leaves of 2 and 3-week-old plants and young inflorescence (YI). EF1 was used as a control for 25 cycles of reaction.
**Supplementary Fig. S5.** The expression patterns of ZAT1p:GUS in the various mutant backgrounds.

(A-B) A transgenic plant (Col-0) harboring ZAT1p:GUS. The ZAT1p:GUS expression was observed in the vasculature together with the root cap (A) and endodermal layer (B). (C) drd2-D overexpressing ZAT1. (D) Q2610>>SMB. A Q2610 line overexpressing SMB by 5x UAS tag localized at the 102 bps upstream of SMB leading to the transition of epidermis into root cap-like cells, an unpublished line. (E) A transgenic plant (Col-0) harboring ZAT1p:GUS observed with Normarski optics. (F) tpst-1 harboring ZAT1p:GUS observed with Normarski optics displaying the multi-layer of ZAT1pGUS expressing root cap cells. Bars = 50 μm.
Supplementary Fig. S6. RT-PCR analysis of ZAT1, ZAT4 and ZAT9 expression in zat1, zat4, zat9, and zat1 zat4 zat9 and the root phenotypes of zat1 zat4 zat9.

(A) RT-PCR analysis of ZAT1, ZAT4, and ZAT9 was performed using total RNA isolated from 5-d-old seedlings of the T-DNA insertion mutants. EF1 was used as a control. (B-C) Root phenotypes WT (B) and zat1 zat4 zat9 (C) seedlings. Bars = 0.5 mm. (D-E) Root meristem phenotypes of WT (D) and zat1 zat4 zat9 (E) imaged with differential interference contrast optics. Asterisks indicate quiescent centers. Bars = 50 μm.