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An *Arabidopsis SUPERMAN*-like gene, *AtZFP12*, Expressed at Shoot Organ Boundaries Suppresses cell Growth.

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***Arabidopsis SUPERMAN* (*SUP*) and its family members have been implicated in flower organogenesis and plant morphogenesis via the regulation of division or growth of cells. In this study, we characterized a new *SUP*–like zinc finger gene (*AtZFP12*). This gene is expressed around the bases of the axillary buds and at the junction between the inflorescence axis and flower stalks. It is also expressed at the boundary between the meristematic and elongation zones in root tips. Overexpression of its cDNA in transgenic *Arabidopsis* reduced cell expansion, resulting in dwarfed plant growth. These results suggest the potential role of *AtZFP12* in the regulation of cell growth during the establishment of SOB in the shoot and transition zones in root tips.**

Key words: lateral organ boundary, SUPERMAN, transcription factor, zinc finger DDBJ/EMBL/GenBank database Accession no. DQ056622

The shoot organ boundaries (SOB) between the shoot meristem and organ primordia, as well as those between adjacent organs, play critical roles in the proper development of plant organs and consequently, in the formation of whole plant architecture (Aida and Tasaka, 2006, 2006). Generally, the cells in the SOB display arrested or reduced division and growth (Aida and Tasaka, 2006). SOB also plays a role as a barrier for defining gene expression patterns (Sakai et al., 1995; Krizek et al., 2006) and controlling the lateral movement of some molecules across the boundary (Rinne and van der Schoot, 1998).

Several genes involved in SOB establishment have been identified, including *SUPERMAN (SUP)* (Bowman et al., 1992; Sakai et al., 1995) and its family members such as *RABIT EARS (RBE)* in *Arabidopsis* (Takeda et al., 2004), *LATERAL SHOOT INDUCING FACTOR (LIF)* in petunia (Nakagawa et al., 2005), and *ramosa 1 (ra1)* in maize (Vollbrecht et al., 2005). These genes (*SUP* family) are unique to the plants and encode proteins containing a single C_2H_2-type zinc-finger motif and an EAR motif-like transcriptional repression domain in their carboxy termini. *SUP* is expressed in the inner domain of floral whorl 3 (stamen whorl) primordia, where it negatively regulates cell proliferation, thereby maintaining the boundary between whorls 3 and 4 (carpel whorl) (Bowman et al., 1992; Sakai et al., 1995). It is suggested that the *SUP*-controlled balance of cell proliferation activity in whorls 3 and 4 is required for restricting *APETALA 3 (AP3)* and *PISTILLATA (PI)* expression to whorls 2 (petal whorl) and 3 (Bowman et al., 1992; Sakai et al., 1995; Sakai et al., 2000). *RBE* is expressed in petal primordia and their precursor cells, where it restricts *AGAMOUS (AG)* expression to whorls 3 and 4 during floral development, such that *RBE* maintains the boundaries between whorls 2 and 3 and those between the individual sepal primordia within whorl 1 (sepal whorl) (Takeda et al., 2004; Krizek et al., 2006). *LIF* and *ra1* have been implicated in controlling the lateral shoot branching and floral branching, respectively (Nakagawa et al., 2005; Vollbrecht et al., 2005). *LIF* is expressed at the basal domain of axillary buds during the vegetative phase of petunia development (Nakagawa et al., 2005), and *ra1* is expressed at the junctions between the spikelet pair meristems and in the primary inflorescence axis of tassels in maize (Vollbrecht et al., 2005).

In this study, we performed functional analysis of a new *Arabidopsis SUP*–like gene encoding zinc finger protein, named *AtZFP12*, according to the naming system of Tague and Goodman (1995) and Dinkins et al. (2002, 2003). We show that *AtZFP12* is expressed around the bases of axillary buds and at the junction between the inflorescence axis and flower stalks. It is also expressed in root tips at the boundary between the meristematic and elongation zones. Overexpression of its cDNA reduced cell growth, resulting in dwarfed plant growth, a distinctive phenotype from those of the other SOB-related *SUP* family genes *SUP*, *RBE* and *LIF*, which are involved in the negative regulation of cell division. These data suggest the potential role of *AtZFP12* in the regulation of cell growth during the establishment of SOB in shoot and transition zones in root tips.

MATERIALS AND METHODS

Plasmid DNA Constructions

For construction of *35S::AtZFP12*, a DNA fragment of the *AtZFP12* cDNA containing a 10-bp untranslated region upstream of a coding sequence (0.45 Kb) was amplified by PCR using *Arabidopsis* genomic DNA as a template. The amplified product was inserted into pBINPLUS-PT (Nakagawa et al., 2005) by using the *Xba*I and *Sac*I sites to yield pBINPLUS-AtZFP12. A *Hind*III-*Eco*RI fragment containing the *CaMV 35S* promoter, *AtZFP12*, and the *nopaline synthase* terminator (*Nos*T) sequences was excised from the resultant pBINPLUS-*AtZFP12* and inserted into the pCAMBIA 1300 vector by using the same sites to yield *35S::AtZFP12* (pCAM-*AtZFP12*). The sequences of the forward and reverse primers used for PCR are 5′-ACGGGATTCTATTCTATATATGGAAAACATCAA AAA-3′ and 5′-ACGGGTACCCTATGTTGTCTTCTTCTTTACGACGCT-3′, respectively; the endonuclease restriction sites are underlined.

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For construction of *AtZFP12* (UC)::*GUS*$^+$, a 3.82-kb fragment containing the upstream (3.37 kb) and full-length coding sequences of *AtZFP12* (UC) with the *attB1* and *attB2* recombination sites at the 5′- and 3′-termini, respectively, was amplified by PCR using *Arabidopsis* genomic DNA as a template. The resulting PCR products were sequentially inserted into pDONR221 by BP reaction and the pCAM-DesGUS$^+$ (Jiang et al., 2008) by LR reaction by using the Gateway system (Invitrogen) to yield a translational fusion product of *AtZFP12* and GUS$^+$. The sequences of the forward and reverse primers used for PCR are as follows: 5′-GGGGACAAGTTTGTACAAAAA-AGCAGGCTTCGGATAAGAAAAAAAAGTCGTGGTAGTGTTTCT-3′ and 5′-GGGGAC- CACTTTGTACAAGAAAGCTGGGTCCG-TACTTGTACTTGTTGTTTTTTTATAAGGA-3′, respectively; the recombination sites are underlined.

For subcellular localization of *AtZFP12*, a PCR-amplified fragment with the full-length *AtZFP12*-coding sequence was inserted into the pCAMBIA1305.1 vector by using the *Nco*I sites to yield a translational fusion product of *AtZFP12* and *GUS*$^+$. The sequences of the forward and reverse primers used for the PCR are as follows: 5′-ACGCCATGGTATTC-TATATATGGAAAACATCAA AAA-3′ and 5′-ACGCCATTGC-TATGTTGTCTTCTTCTTTACGACGCT-3′, respectively; the endonuclease restriction sites are underlined.

RNA Analyses

Total RNA was isolated from various vegetative and floral organs of the *Arabidopsis* plant using the RNeasy Plant Kit (Qiagen, Hilden, Germany) and used for reverse transcription PCR (RT-PCR) and RNA gel-blot hybridization as described previously (Jiang et al., 2008). The sequences of primers used for PCR amplification of *AtZFP12* sequence were 5′-GCGAACCTAAAAGAAGAAGACAGTGAAGAT-3′ and 5′-CACGTCTGCACTTTGAGATAAAGCCTTTTG-3′.

GUS Staining and Microscopic Observation

GUS staining and microscopic observation were performed as described previously (Jiang et al., 2008). For the subcellular localization of the *AtZFP12*-GUS fusion protein, onion epidermis was fixed at 24 h after biolistic delivery of *35S*:: *AtZFP12-GUS* DNA, GUS stained, and observed under a microscope (Leica DMR, Welzlar, Germany) with Nomarski optics. For the histochemical analysis of *AtZFP12* promoter activity, the *AtZFP12* (UC)::*GUS*$^+$ transgenic *Arabidopsis* plants were fixed, GUS stained, and viewed under a stereo microscope (Leica MZFL III). The GUS-stained plant tissues were also processed for paraffin sectioning, fixed in FAA (3.7% formaldehyde, 50% ethanol and 5% glacial acetic acid), dehydrated in ethanol/butanol series, and embedded in paraplast (Sigma). Serial 10 μm sections were prepared, deparaffinized, and observed under a microscope (Leica DMR, Leica Microsystems, Welzlar, Germany).

RESULTS AND DISCUSSION

AtZFP12 Encodes a Nuclear SUP-like Zinc Finger Protein

AtZFP12 encodes a protein containing a single C_2H_2-type zinc finger and an EAR-like transcriptional repression domain.

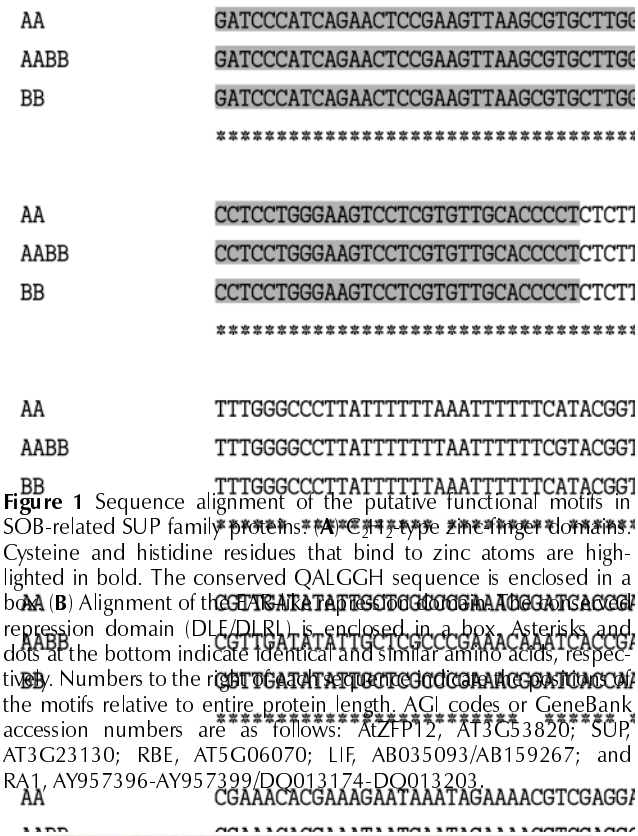

Figure 1 Sequence alignment of the putative functional motifs in SOB-related SUP family proteins. (**A**) C_2H_2-type zinc finger domains. Cysteine and histidine residues that bind to zinc atoms are highlighted in bold. The conserved QALGGH sequence is enclosed in a box. (**B**) Alignment of the EAR-like repression domain. Asterisks and dots at the bottom indicate identical and similar amino acids, respectively. Numbers to the right indicate the position of the motifs relative to entire protein length. AGI codes or GeneBank accession numbers are as follows: AtZFP12, AT3G53820; SUP, AT3G23130; RBE, AT5G06070; LIF, AB035093/AB159267; and RA1, AY957396-AY957399/DQ013174-DQ013203.

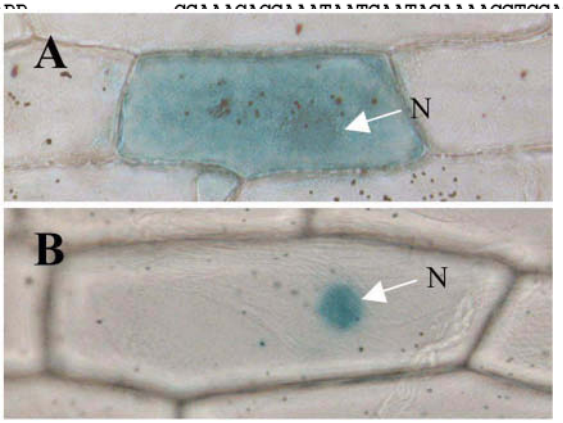

Figure 2 Subcellular localization of the AtZFP12-GUS$^+$ protein. Onion epidermis was bombarded with the DNA constructs *35S*::*AtZFP12-GUS*$^+$ (**A**) and *35S*::*GUS*$^+$ (**B**, negative control). After overnight incubation at 25°C, subcellular localization of GUS activities was examined under a microscope. Arrows indicate the nuclei (N).

As shown in figure 1, The protein encoded by *AtZFP12* shares a high degree of homology with the other SUP-family members, which have been implicated in SOB establishment, in both the zinc-finger motif (A) and the EAR-like transcriptional repression domain (B). It has been demonstrated that the zinc-finger domain of SUP specifically binds to the DNA with a core sequence of AGT (Dathan et al., 2002), and that the hexapeptide DLELRL has a strong transcriptional repression activity (Hiratsu et al., 2002; Hiratsu et al., 2004). Transient expression of the GUS fusion protein in onion epidermis revealed that the GUS$^+$ activity is localized in the nucleus, indicating that *AtZFP12* encodes a nuclear

protein (Figure 2A). In comparison, GUS$^+$ alone was distributed throughout the cells (Figure 2B). These data are consistent with AtZFP12 protein being a transcription factor, more specifically a transcriptional repressor, although further studies are needed to conclude.

AtZFP12 Overexpression Suppressed Cell Growth

We obtained 12 lines of transgenic *Arabidopsis* plants overexpressing *AtZFP12* (*AtZFP12*-ox) and most of them exhibited similar phenotypes. As shown in figure 3A-b, *AtZFP12* overexpression resulted in severely dwarfed plant growth. In *AtZFP12*-ox flowers, the growth of petals and stamens was suppressed, while that of sepals and pistils was nearly normal both in size and morphology (Figure 3A-d). These phenotypes of *AtZFP12-ox Arabidopsis* were similar to those of *SUP*-ox *Arabidopsis*, with the exception that growth of the sepals was also suppressed in the latter (Hiratsu et al., 2002; Yun et al., 2002). Microscopic observation of epidermal cells in inflorescence stems revealed that the size of cells in *AtZFP12*-ox plants was drastically reduced (Figure 3B-b, 3C) in comparison to that in wild-type plants (Figure 3B-a, 3C). This cellular effect of *AtZFP12* overexpression suggests that *AtZFP12* is predominantly involved in the negative regulation of cell growth, although these results do not exclude its possible function in cell division control. It is interesting to note that despite the similarity in protein sequences as well as the expression patterns in the lateral organ boundaries, the cellular function of *AtZFP12* deduced from the overexpression phenotype was obviously different from *LIF*, whose overexpression in petunia decreased cell number with accompanying compensatory enlargement in cell size (Nakagawa et al., 2005).

AtZFP12 is Expressed in SOB

RT-PCR was employed to determine *AtZFP12* expression in 2-week-old whole seedlings (SD), root tips (RT), rosette leaves (RL), cauline leaves (CL), inflorescence stems (ST), inflorescence nodes containing cauline axils (AX), floral nodes (FN) and flowers (FL) of *Arabidopsis* plants. As shown in figure 4A, relatively high levels of *AtZFP12* transcripts were detected in roots (RT), cauline axils (AX) and floral nodes (FN).

We further analyzed the spatiotemporal pattern of *AtZFP12* expression in *Arabidopsis* plants using *AtZFP12* (UC)::*GUS*$^+$ transgenic plants (Figure 4B). GUS staining of the shoots was observed at the basal domains of axillary buds (Xb) in the axils (Ax) of both rosette (Figure 4B-c) and cauline leaves (Figure 4B-a, b). A closer observation after detachment of an axillary bud from the axil with a forceps revealed a donut-shaped pattern of GUS stain in the axil (Figure 4B-d), demonstrating that *AtZFP12* is expressed surrounding the bases of axillary buds. GUS staining was also observed at the junction between the inflorescence axis and flower stalks (FN, Figure 4B-a). Thin paraffin sections of the GUS-stained plant tissues further confined the *AtZFP12* expression to a small population of cells around the base of axillary bud (Figure 4B-e) and to those at the junction between the inflorescence stem and floral stalk (Figure 4B-f). These observations are corroborative of the RT-PCR results

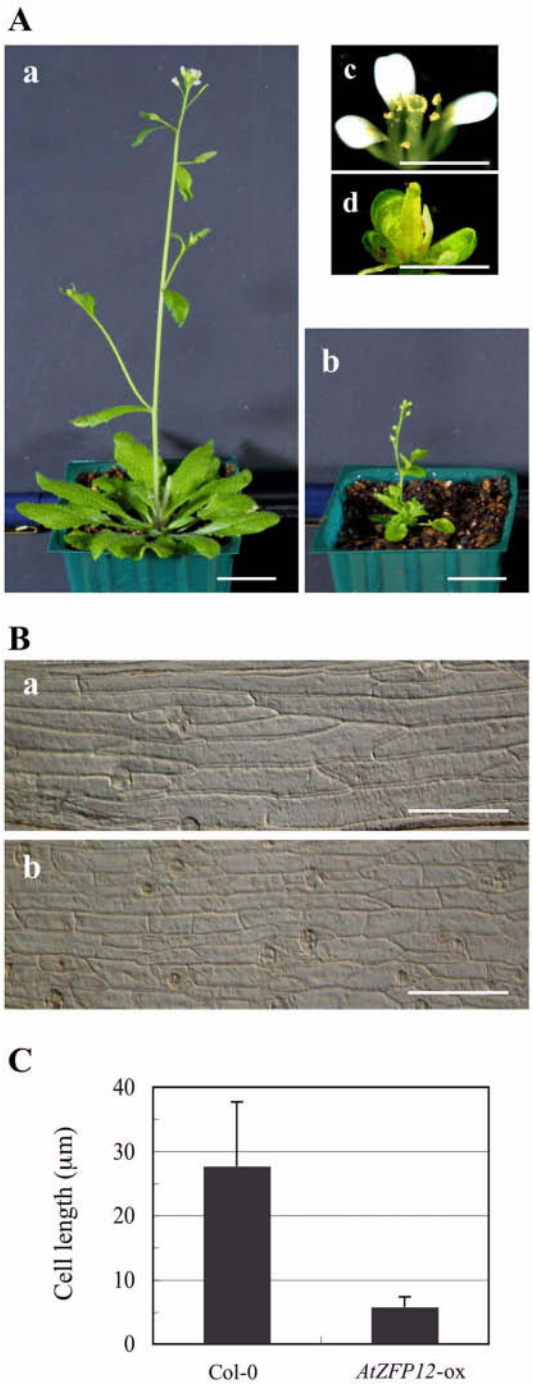

Figure 3 Overexpression of *AtZFP12* in *Arabidopsis*. **(A)** Phenotype of *AtZFP12*-ox line (b and d) grown for 4 weeks after sowing. (a) and (c), wild-type plant (Col-0). (**B**) Microscopic views illustrating epidermal cells of inflorescence stems of Col-0 (a) and *AtZFP12*-ox (b) plants. (**C**) A bar graph showing average cell lengths of inflorescence stems in wild type (Col-0) and *AtZFP12*-ox plants. Averages of 50 cells from 4 plants are shown with standard deviations. Scale bars: 2 cm in A-a and A-b, 2 mm in A-c and A-d, and 10 μm in B.

described above. Taken together with the effect on cell growth (Figure 4B-b), the specific expression pattern at SOB in the shoot (Figure 4B) implicates the potential role of *AtZFP12* in SOB establishment through suppression of cell growth during plant development.

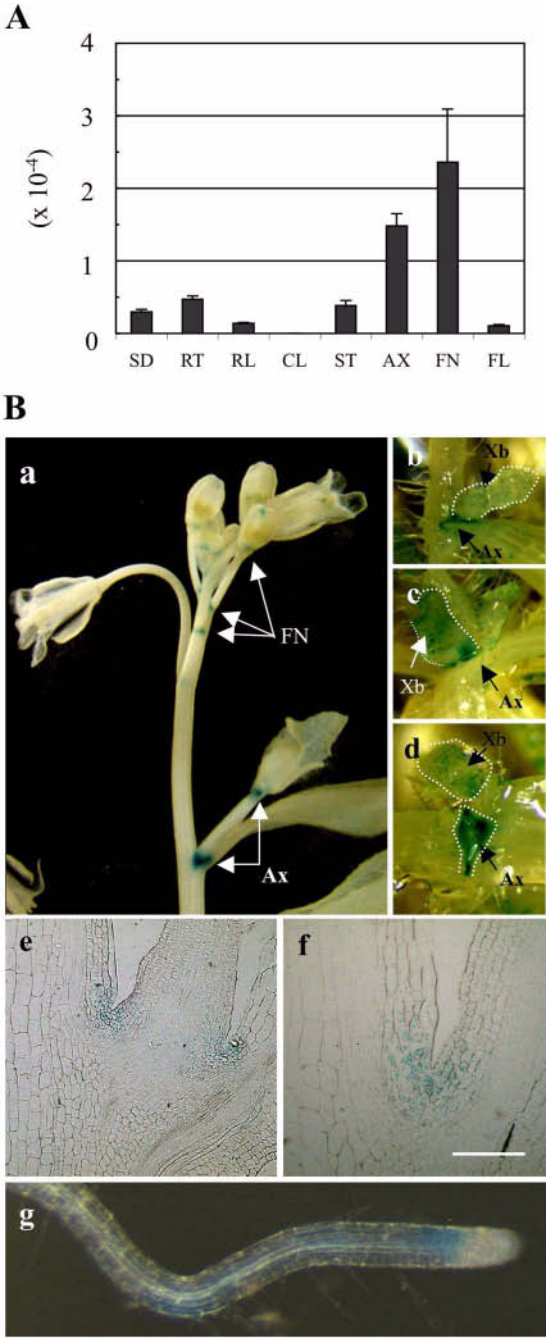

Figure 4 Expression patterns of *AtZFP12*. (**A**) RT-PCR analysis using total RNA from 2-week-old whole seedlings (SD), root tips (RT), rosette leaves (RL), cauline leaves (CL), inflorescence stems (ST), inflorescence nodes containing cauline leaf axils (AX), floral nodes (FN), and flowers (FL). Averages of three determinations relative to those of *ACT2* are shown. (**B**) Histochemical analysis of GUS activities in the shoot (a-f) and root (g). Transgenic *Arabidopsis* plants bearing *AtZFP12* (UC)::*GUS*$^+$ chimeric gene were stained for GUS activity and observed under a dissection microscope. GUS staining was observed at the basal domains of axillary buds (Xb) in the axils (Ax) of both cauline (a and b) and rosette leaves (c), and at the junction between the inflorescence axis and flower stalks (FN, a). Donut-shaped pattern of GUS stain in the axil is seen in (d). (e) and (f) illustrate longitudinal sections showing GUS activities in cells around the base of an axillary bud (e) and at the junction between inflorescence stem and a floral stalk (f). Ax: axil; Xb: axillary bud; FN: floral node.

The pattern of *AtZFP12* expression in the axils of leaves is similar to those of *Arabidopsis LATERAL ORGAN BOUNDARIES* (*LOB*) (Shuai et al., 2002), and petunia *LIF* (Nakagawa et al., 2005). It would be interesting to investigate the possible genetic interactions among these genes.

Expression of *AtZFP12* in Roots

In the roots, 3 distinct but overlapping zones can be identified along the longitudinal axis of root tips: the apical meristematic, elongation, and differentiation (maturation) zones (Dolan et al., 1993). Cells proliferate in the meristematic zone and elongate primarily in the elongation zones (van der Weele et al., 2003). Figure 4B-g illustrates that the GUS activity in *AtZFP12* (UC)::*GUS*$^+$ transgenic plants is localized at the boundary region between meristematic and elongation zones of the root tips. GUS staining was also observed in mature roots in an irregular and uneven pattern (Figure 4B-g). A similar expression pattern was observed for *KNAT5*, i.e., *KNAT5* promoter-driven GFP marked the boundary between the dividing and elongating epidermal cells (Truernit et al., 2006). These expression patterns may imply certain roles of these genes in the regulation of transition from cell division to cell elongation, characteristic of meristematic and elongation zones during root development.

To characterize the loss-of-function phenotype of *AtZFP12*, we obtained a hemizygous T-DNA insertion mutant line from the Salk Institute collections (Alonso et al., 2003) and self-pollinated it to yield a homozygous knockout plants. However, no appreciable changes in root growth, shoot morphology, and floral organogenesis were detected in this mutant line (data not shown). This may suggest some functional redundancy of *AtZFP12* with the other gene(s) in *Arabidopsis*. Simultaneous mutations with other gene(s) such as *LOB*, may aid in further elucidating the function of *AtZFP12* in plant morphogenesis.

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