Journal of Experimental Botany, Vol. 62, No. 13, pp. 4571–4581, 2011
doi:10.1093/jxb/err168 Advance Access publication 30 May, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Overexpression of EVE1, a novel ubiquitin family protein, arrests inflorescence stem development in *Arabidopsis*

Hyun-Ju Hwang*, Hoyeun Kim*, Young-Min Jeong, Monica Y. Choi, So-Young Lee and Sang-Gu Kim†

Department of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: kimsg@snu.ac.kr

Received 22 March 2011; Revised 22 March 2011; Accepted 28 April 2011

Abstract

In *Arabidopsis*, inflorescence stem formation is a critical process in phase transition from the vegetative to the reproductive state. Although inflorescence stem development has been reported to depend on the expression of a variety of genes during floral induction and repression, little is known about the molecular mechanisms involved in the control of inflorescence stem formation. By activation T-DNA tagging mutagenesis of *Arabidopsis*, a dominant gain-of-function mutation, *eve1-D* (*eternally vegetative phase1-Dominant*), which has lost the ability to form an inflorescence stem, was isolated. The *eve1-D* mutation exhibited a dome-shaped primary shoot apical meristem (SAM) in the early vegetative stage, similar to that seen in the wild-type SAM. However, the SAM in the *eve1-D* mutation failed to transition into an inflorescence meristem (IM) and eventually reached senescence without ever leaving the vegetative phase. The *eve1-D* mutation also displayed pleiotropic phenotypes, including lobed and wavy rosette leaves, short petioles, and an increased number of rosette leaves. Genetic analysis indicated that the genomic location of the *EVE1* gene in *Arabidopsis thaliana* corresponded to a bacterial artificial chromosome (BAC) F4C21 from chromosome IV at ~17cM which encoded a novel ubiquitin family protein (At4g03350), consisting of a single exon. The EVE1 protein is composed of 263 amino acids, contains a 52 amino acid ubiquitin domain, and has no glycine residue related to ubiquitin activity at the C-terminus. The *eve1-D* mutation provides a way to study the regulatory mechanisms that control phase transition from the vegetative to the reproductive state.

Key words: *Arabidopsis* development, bolting, inflorescence stem, phase transition, shoot apical meristem, ubiquitin family protein.

Introduction

The shoot apical meristem (SAM) generates all plant parts that appear above the ground, including the shoot system (rosette leaves and inflorescence stem) and flowers. In *Arabidopsis*, the SAM undergoes several transitions throughout its lifetime. One significant transition is the conversion from vegetative to reproductive growth. In this phase transition, the SAM switches to an inflorescence meristem (IM). Subsequently, the IM produces a floral meristem (FM) as it enters the reproductive phase of growth (Reddy and Meyerowitz, 2005). This transition is marked by the formation of an inflorescence stem, a critical time point at which observable morphogenetic events take place. Much progress has been made in understanding the phase transition from the vegetative to the reproductive state. Thus, the phase transition is precisely demonstrated by coordinating the response to environmental factors (day length, light intensity, temperature, etc.) and endogenous changes such as phytohormones or the regulation of flowering genes (Baurle and Dean, 2006). However, the events involved in inflorescence stem formation have remained largely uncharacterized.

Cellular and genetic analyses of inflorescence stem formation have been described in a few mutants. The recessive strong *shootmeristemless* (*stm*) alleles are unable to maintain the SAM and terminate development in the seedling state (Endrizzi *et al.*, 1996; Long *et al.*, 1996). STM

is a homeodomain transcription factor of the KNOTTED-like homeobox (*KNOX*) class and promotes SAM identity. STM is required not only for the initiation of the shoot meristem during embryogenesis but also for subsequent maintenance of the vegetative SAM, IM, and FM (Clark *et al.*, 1996; Long *et al.*, 1996; Lenhard *et al.*, 2002). Another class-1 *KNOX* gene, *KNAT1/BP*, plays a key role in the development of the SAM and the inflorescence stem. The overexpression of *KNAT1/BP* activated ectopic SAM formation and a loss-of-function mutation resulted in reduced floral internodes (Lincoln *et al.*, 1994; Chuck *et al.*, 1996; Douglas *et al.*, 2002; Venglat *et al.*, 2002). The *Arabidopsis* primary inflorescence-deficient mutant, *sha1-1*, shows normal primary SAM development in the juvenile vegetative stage, but the SAM becomes dysfunctional after entering the adult vegetative stage. The *SHA1* gene, which encodes a RING finger E3 ligase, is required for post-embryonic SAM maintenance through effects on the *WUSCHEL* (*WUS*) signalling pathway (Sonoda *et al.*, 2007). To our knowledge, the mechanism of gene regulation associated with inflorescence stem formation (bolting) during phase transition in *Arabidopsis* is still unclear.

To better understand the molecular mechanisms that control phase transition, it is useful to isolate mutants that affect transition from the vegetative to the reproductive phase of growth. In this study, a new dominant mutant, *eve1-D*, associated with defective inflorescence development was isolated. The *eve1-D* mutation resulted in the over-expression of a novel ubiquitin family protein (EVE1). It is proposed that the EVE1 protein may play a critical role in inflorescence stem formation during phase transition in the development of *Arabidopsis*.

Materials and methods

Isolation and characterization of the mutant

Arabidopsis (*A. thaliana*) ecotype Columbia-0 plants were transformed with pSKI015 using the floral dip method (Clough and Bent, 1998; Weigel *et al.*, 2000) and screened for mutations resulting in abnormal phenotypes. T-DNA-tagged plants were selected by spraying with 0.1% Basta (Duchefa) twice a week for 3 weeks. All *Arabidopsis* plants were grown in long days (16 h light/8 h dark) under fluorescent lights at 22 °C with 70% humidity.

To clone the T-DNA-inserted genomic sequences, the plasmid rescue technique was applied (Medford *et al.*, 1992). The recovered plasmids from *Eco*RI-digested genomic DNA isolated from *eve1-D* plants were analysed further. The genomic fragments containing the T-DNA were rescued by spreading on Luria–Bertani (LB) agar plates containing ampicillin. A T-DNA primer close to the T-DNA left border was used to sequence the adjacent genomic sequences. BLASTN was used to localize the insertion positions in the *Arabidopsis* genome using the National Center for Biotechnology Information (NCBI) *A. thaliana* genome database.

Complementation test and generation of transgenic antisense lines

The sense and antisense constructs of the *EVE1* gene were created by PCR amplification of the genomic DNA from the 5′-upstream region of *EVE1* to the stop codon of *EVE1*. The primers used to generate the *EVE1* ORF (open reading frame) were 5′-AA**GGTACC**GTTTGATCACTAATCG-3′ and 5′-AA**CTGCAG**CTCACTTCTCACGGAT-3′ (restriction sites are shown in bold, and the sequence corresponding to *EVE1* is underlined), which generated a 1.3kb fragment that was digested with *Pst*I and *Sal*I and ligated into the *Pst*I and *Sal*I sites of pMN20 for complementation. For transgenic antisense lines, the primers used to generate the *EVE1* ORF were 5′-GG**GAATCC**ACGTTTGATCACTA-3′ and 5′-AA**GAATTC**TAACCGTCGATT-3′. The PCR product was digested with *Bam*HI and ligated into the *Bam*HI sites of the binary vector pBI121 in antisense orientation. Transgenic plants were generated in the wild type by floral dipping and selected by 50mg l^{-1} kanamycin.

Real-time PCR and RT-PCR analysis

Total RNA was extracted from shoot apices of 2-week-old plants using the Tri reagent (Sigma) according to the manufacturer's instructions. The real-time PCR was performed either on a StepOne Real Time PCR System (Applied Biosystems) or by using the comparative CT (ΔCT) method with 1× SYBR green PCR master mix (Applied Biosystems). Negative controls were performed by using the same reaction mixtures without cDNA. The gene expression levels were normalized to *β-tubulin* gene (*β-TUB*) expression levels. The gene-specific primers are described in Supplementary Table S2 available at *JXB* online. For RT-PCR, total RNA extracted from various tissues of wild-type and *eve1-D* mutant plants was isolated and reverse transcribed using an RT-PCR kit (Takara). The RT-PCR experiment was performed using three independent RNA samples.

Histology and microscopy

To obtain cross-section and scanning electron microscopy (SEM) images of SAM, samples were placed in a fixation solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) under vacuum conditions for 2 d at 4°C. Each sample was prepared by methods described previously (Lee *et al.*, 2010).

Phylogenetic analysis

Nucleotides and predicted amino acid sequences of ubiquitin family proteins in *Arabidopsis* were obtained from GenBank. Distance trees were constructed using the Neighbor–Joining (NJ) method, implemented using the NEIGHBOR program in BIOLOGY WORKBENCH (http://www.workbench.sdsc.edu).

Nuclear localization of EVE1–GFP fusion protein

To make an EVE1–green fluorescent protein (GFP) fusion protein, the *EVE1* cDNA sequence was amplified by PCR using the G-F (5′-AAGGATCCAAATGAACGTGGACATC-3′) and G-R (5′-TTGGATCCTCACTTCTCACGGATA-3′) primers containing a *Bam*HI site and then fused to GFP. Rosette leaves of 2-week-old wild-type plants were used for the isolation and transformation of protoplasts. A 10 μg aliquot of plasmid DNAs containing EVE1–GFP fusion constructs was transfected into the protoplasts. Then, protoplasts were incubated in dark conditions at 24 °C for 24h. Images were obtained using a confocal microscope (Bio-rad, Radiance 2000/MP).

Results

The eve1-D *mutation blocks the transition to flowering and alters leaf morphology*

To investigate the molecular mechanism of inflorescence stem development, screening was carried out to look for a mutant from the activation T-DNA treatment that did not generate the inflorescence stem. The SAM of the mutant

plants did not convert to IM and remained indefinitely as SAM, characteristic of the vegetative phase of the growth, so the mutation was named *eve1-D* (for *eternally vegetative phase1-Dominant*). At the early seedling stage, the *eve1-D* plants exhibited small cotyledons with short petioles. The emerged rosette leaves of *eve1-D* plants were smaller than those of wild-type plants (Fig. 1A D,). During the vegetative stage of growth, *eve1-D* plants displayed lobed and wavy rosette leaves with short petioles (Fig. 1B, C, E, and F). Wild-type plants generally began to bolt at 20 days after germination (DAG) and showed a primary inflorescence, secondary inflorescence, and flowers at 25 DAG. However, *eve1-D* plants showed only the rosette leaves of the vegetative phase and did not generate the primary inflorescence (Fig. 1G, I). After 40 DAG, wild-type plants generated axillary and lateral inflorescences with siliques, but *eve1-D* plants failed to produce the primary, axillary, and lateral inflorescences, and remained vegetative (Fig. 1H, J).

The leaves of wild-type and *eve1-D* plants exhibited characteristic differences. The length of rosette leaves in *eve1-D* plants was ~60% that of wild-type leaves, and their petioles were ~40% of the size of the wild-type petioles (Table 1). Although the juvenile leaf number in *eve1-D* plants and wild-type plants was similar, the number of adult rosette leaves formed in *eve1-D* plants was much greater than in wild-type plants (Table 1, Fig. 2A, B). The wavy margins of *eve1-D* plants appeared from the basal part of young leaves (Fig. 2B). SEM analysis showed that wild-type leaves were flat (Fig. 1C, D), but *eve1-D* leaves exhibited a lobed and outward phenotype (Fig. 1E). In particular, the margins of the *eve1-D* rosette leaves were severely lobed and had a deep sinus shape (Fig. 1F).

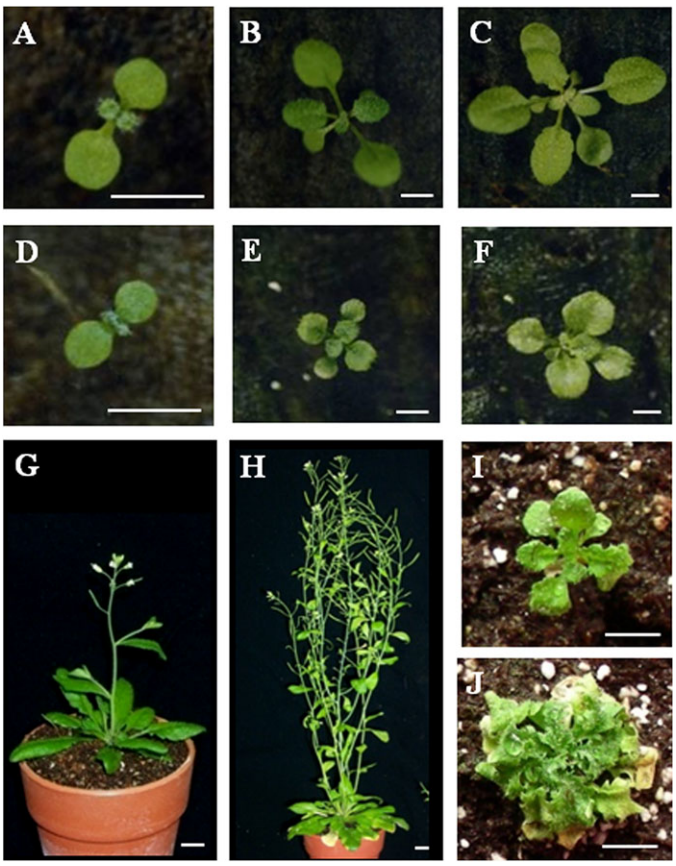

Fig. 1. Comparison of wild-type and *eve1-D* plants at various developmental stages. (A–F) Phenotypes of 5-day-old wild-type (A) and *eve1-D* mutant (D) plants, 10-day-old wild-type (B) and *eve1-D* mutant (E) plants, and 15-day-old wild-type (C) and *eve1-D* mutant (F) plants. (G) A 25-day-old wild-type plant. (H) A 40-day-old wild-type plant. (I) A 25-day-old *eve1-D* plant. (J) A 40-day-old *eve1-D* plant. Bars=100mm in A–J.

The structures of the SAMs in wild-type and *eve1-D* plants were compared in detail at several developmental stages (Fig. 2G–L). Fifteen-day-old wild-type plants showed normal dome-shaped IM and FM at the same time (Fig. 2G–I). However, 25-day-old *eve1-D* plants exhibited only the dome-shaped SAM (Fig. 2J–L). Histological analysis showed that wild-type plants displayed the dome-shaped SAM at 10 DAG (Fig. 3A), and IM, flowers, axillary SAMs, and FMs at 20 DAG (Fig. 3B). However, the *eve1-D* plant showed only dome-shaped SAM at 10 and 20 DAG (Fig. 3C, D). After 40 DAG, *eve1-D* plants displayed axillary SAMs, but these still remained dome-shaped (Fig. 3E). Even though the *eve1-D* plant showed axillary and lateral SAMs, they did not display axillary or lateral inflorescences (Fig. 3E, F).

eve1-D/+ *plants exhibit defective stem development*

Since the *eve1-D* mutation arrested development at the vegetative stage of growth, *eve1-D*/+ plants were obtained to examine the effects of this mutation further. The *eve1-D*/+ plants exhibited a loss of apical dominance, late flowering, and a dwarf phenotype (Fig. 4, Supplementary Table S1 at *JXB* online). The rosette leaves in *eve1-D*/+ mutants displayed a severely wavy and lobed phenotype (Fig. 4A, B, E, F) and were curled, in contrast to wild-type leaves in longitudinal section (Supplementary Fig. S1A, B). The leaf number and size were almost similar to those of *eve1-D* (Table 1). In the adult vegetative stage, the *eve1-D*/+ plants produced a primary inflorescence with reduced length of the internode and continued to produce axillary and lateral inflorescences (Fig. 4C–G). The lengths of inflorescence stems

Table 1. Morphological analysis of wild-type, *eve1-D*/+, and *eve1-D* leaves

		Wild type	*eve1-D*/+	*eve1-D*
No. of leaves[a]	Juvenile	4.7±0.5	6.5±0.5	4.9±1.3
	Adult	7.0±0.4	13.4±1.3	15.2±9.6
	Cauline	3.7±0.3	8.2±1.1	ND
Size of rosette leaf[b]	Length	3.1±0.3	2.8±0.3	2.3±0.2
	Width	1.4±0.2	1.0±0.2	1.0±0.3
Length of petiole		1.0±0.3	0.5±0.02	0.4±0.02

[a] Juvenile rosette leaves lacked trichomes on the adaxial surface, whereas adult rosette leaves had trichomes on the adaxial surface. Cauline leaves on the primary inflorescence were included. The values are given as means ±SD, *n*=30. ND, not determined.
[b] Measured on the fifth leaves after bolting.

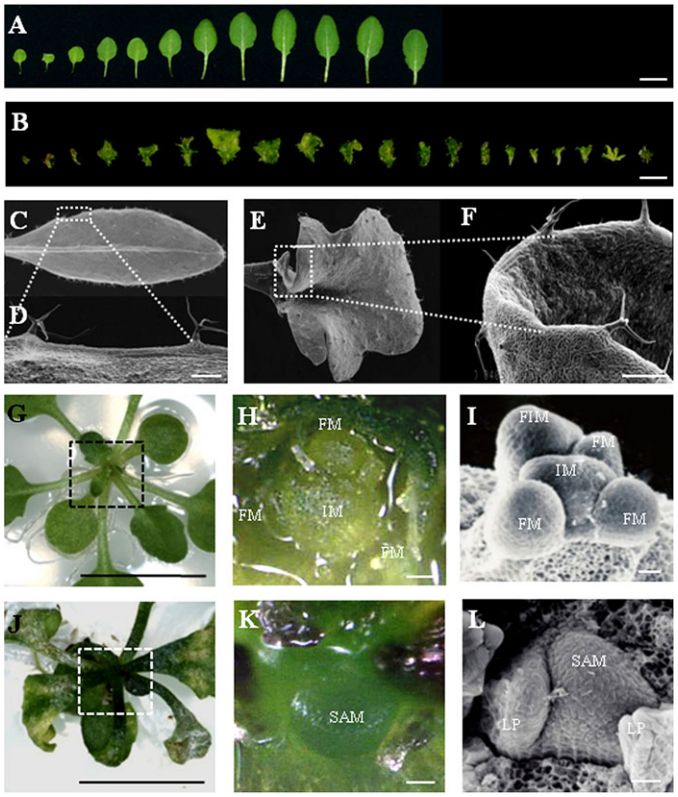

Fig. 2. Comparison of the wild type and *eve1-D* in terms of the leaves and SAM. (A) Rosette leaves of a 25-day-old wild-type plant. (B) Rosette leaves of a 25-day-old *eve1-D* plant. (C–F) Scanning electron micrograph of the leaf of a wild-type (C) and an *eve1-D* (E) plant and close-up of wild-type (D) and *eve1-D* (F) leaves. (G) A 15-day-old wild-type plant. (H) Magnified SAM of a 15-day-old wild-type plant. (I) Scanning electron microscopic observation of the SAM in a 15-day-old wild-type plant. (J) A 25-day-old *eve1-D* plant. (K) Magnified SAM of a 25-day-old *eve1-D* plant. (L) Scanning electron microscopic observation of the SAM in a 25-day-old *eve1-D* plant. IM, inflorescence meristem; SAM, shoot apical meristem; LP, leaf primordia; FM, floral meristem. Bars=100 mm in A, B, G, and J, 10 μM in C–F, and 100 μM in H, I, K, and L.

and internodes in the mature *eve1-D*/+ plants were shorter than those of wild-type plants (Supplementary Table S1). The stem width critically decreased in *eve1-D*/+ plants (Fig. 4H, I, L, M). The epidermal cells of the stem in *eve1-D*/+ plants were slightly shorter and larger than those of the wild type (Fig. 4J, K, N, O). The length of *eve1-D*/+ siliques was shorter than those of wild-type plants (Supplementary Fig. S1C, Supplementary Table S1). The siliques of *eve1-D*/+ plants produced fewer seeds than those of the wild-type plants. However, seed weight remained about the same (Supplementary Fig. S1F, Supplementary Table S1). On dissection, immature siliques of the self-fertilized *eve1-D*/+ plants were found to contain partially aborted seeds, while the siliques of wild-type plants had very low levels of seed abortion (Supplementary Fig. S1D, E). In addition, carpel valves of *eve1-D*/+ plants hardly dehisced at fruit maturation (Supplementary Fig. S1G–J).

The EVE1 *gene encodes a ubiquitin family protein*

To identify the gene responsible for the *eve1-D* mutation, the position of the T-DNA insertion was determined by plasmid rescue (Fig. 5A). Sequence analysis of the rescued plant DNA revealed that the insertion was in the position in the genome represented by the *A. thaliana* bacterial artificial chromosome (BAC) F4C21 from chromosome IV at ~17 cM. The sequences spanned nucleotides 105629–107424 of BAC F4C21 and included the sequences of the ubiquitin family protein (At4g03350, GenBank accession no. NM_116573). The *EVE1* gene encodes a ubiquitin family protein that contains a 53 amino acid ubiquitin domain and consists of a single exon. The full-length *EVE1* cDNA was 792 bp and encoded a protein of 263 amino acids (Fig. 5A, D). The expression levels of the other genes near the T-DNA insert site were determined, including the *EVE1* gene in *eve1-D* plants. Only the *EVE1* gene was increased in *eve1-D* plants. The neighbouring genes near the T-DNA insert site were not affected by an enhancer of T-DNA (Fig. 5B).

Phylogenetic analysis using the ubiquitin domain showed that among ubiquitin superfamilies, such as ubiquitin-like protein (UBLs), ubiquitin, Nedd8, and ANTHOCYANIN1 (AN1), EVE1 is most similar to the RADIATION SENSITIVE 23 (RAD23) protein (At1g79650) in *Arabidopsis*. Ubiquitin is a highly conserved small protein of 76 amino acids in eukaryotes and plays a well-established role in protein degradation. Polyubiquitin chains are covalently attached between the C-terminal glycine residue of ubiquitin and the ϵ-amino group of the substrate lysine, and are targeted as a sign for their recognition and degradation by the 26S proteasome (Hofmann and Pickart, 2001). The amino acid sequence identity in the ubiquitin domain of EVE1 is 78% in comparison with the common ubiquitin domain. The C-terminus of EVE1 lacks the glycine residues that are required for the activation of ubiquitin (Fig. 5C, D).

To investigate the spatial expression patterns of *EVE1* transcripts and proteins in various tissues of plants, RT-PCR and western blot analyses were performed. Total RNA and proteins were isolated from the seedling, roots, stems, rosettes, and flowers. The RT-PCR and western blot analyses indicated that the *EVE1* gene and protein were expressed in all tissues of the wild-type plants (Fig. 6A, B). To examine the subcellular localization of EVE1, GFP was fused to the C-terminus of the *EVE1* gene for expression of the corresponding protein. *Arabidopsis* mesophyll protoplasts were transfected with the GFP construct to transiently express *EVE1–GFP* under the control of the 35S promoter of cauliflower mosaic virus (CaMV). The EVE1 protein was localized in the nucleus (Fig. 6C–J).

Morphologies of the transgenic Arabidopsis *plants expressing sense and antisense* EVE1 *mRNA*

To determine whether increased expression of the *EVE1* gene was capable of causing an abnormality and arresting phase transition to inflorescence stem development, an

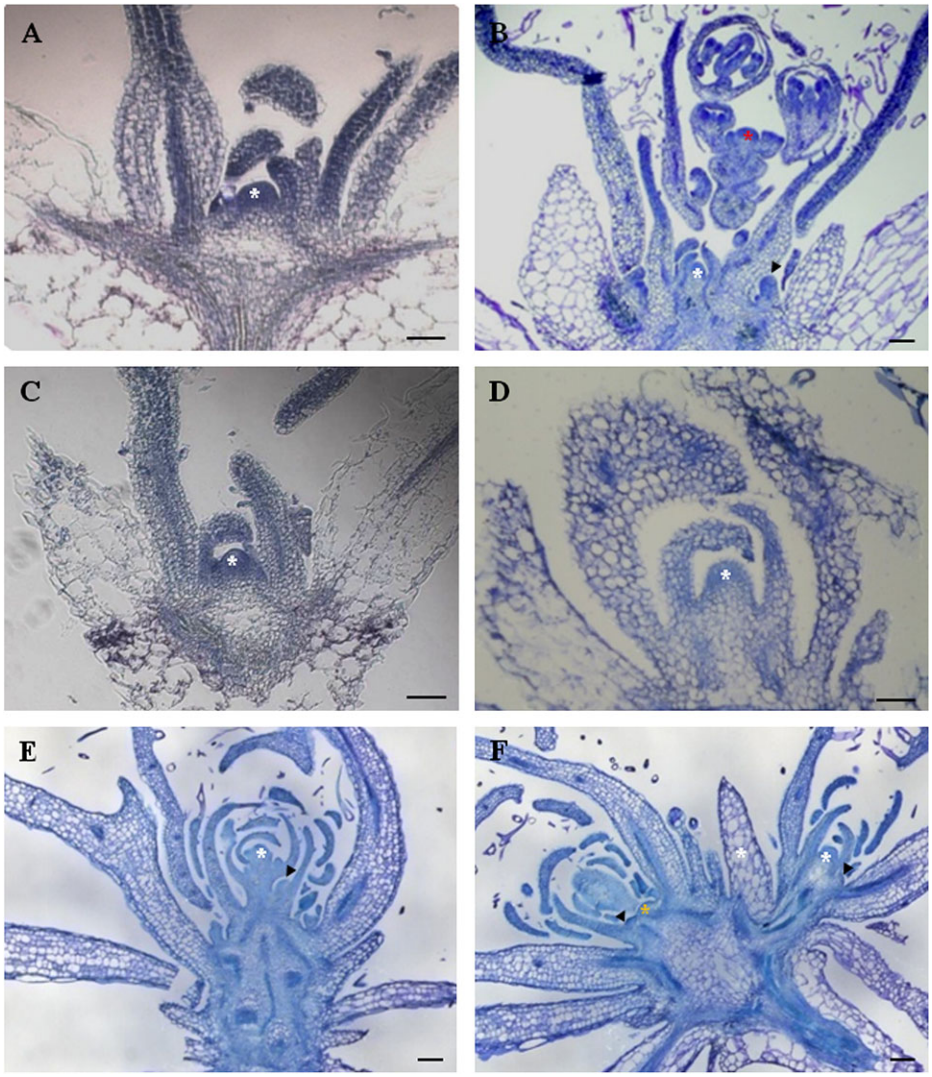

Fig. 3. Longitudinal sections through the SAM of wild-type and *eve1-D* plants. (A) A 10-day-old wild-type plant. (B) A 20-day-old wild-type plant. (C) A 10-day-old *eve1-D* plant. (D) A 20-day-old *eve1-D* plant. (E) A 40-day-old *eve1-D* plant. (F) A 50-day-old *eve1-D* plant. White asterisk, SAM; yellow asterisk, lateral SAM; red asterisk, IM; and black arrowhead, axillary SAM.

attempt was made to recreate the phenotype with a construct designed to increase the expression of the *EVE1* gene (Fig. 7F). Wild-type plants were transformed with a construct harbouring the *EVE1* ORF, including the *EVE1* promoter under the CaMV 35S enhancer tetramer in pMN20 (Weigel *et al.*, 2000). The expression of the *EVE1* gene was highly accumulated in *EVE1*-overexpressing transgenic plants (Fig. 7G). At the young seedling stage, *EVE1*-overexpressing transgenic plants showed lobed rosette leaves (Fig. 7A, B). At 35 DAG, the transgenic plants did not bolt and still remained at the vegetative stage, while the wild-type plants showed inflorescence stems (Fig. 7C, D). Up to 45 DAG, transgenic plants did not produce the inflorescence stem (Fig. 7E). This was sufficient to replicate the *eve1-D* phenotypes.

To determine whether knockout or knockdown mutation may affect the EVE1 phenotype, >100 transgenic *Arabidopsis* plants expressing antisense *EVE1* mRNA in the wild-type plants were generated. All of the transgenic lines showed reduced amounts of antisense *EVE1* mRNA, but the phenotypes were similar to the wild type, as shown in the representative transgenic plants in Supplementary Fig. S2 at *JXB* online.

AP1 *and* AP2 *are down-regulated in the* eve-1D *mutant*

The molecular network affected by the *eve-1D* mutation was investigated using real-time PCR to analyse the transcription levels of the various genes known to be related to SAM development and maintenance. The expression levels of homeodomain genes, such as *WUS*, *WUSCHEL RELATED HOMEOBOX 2* (*WOX2*), and *WOX5*, did not exhibit any differences in wild-type and *eve1-D* plants (Fig. 8A). Similarly, *Arabidopsis* class I *KNOX* genes for SAM development, *STM*, *KNAT1*, *KNAT2*, and *KNAT6*, did not show significant differences in expression levels in wild-type and *eve-1D* plants (Fig. 8B). In relation to leaf polarization, the expression of *KANADI1* (*KAN1*) and *KAN2* genes was analysed and it was found that the expression of these genes

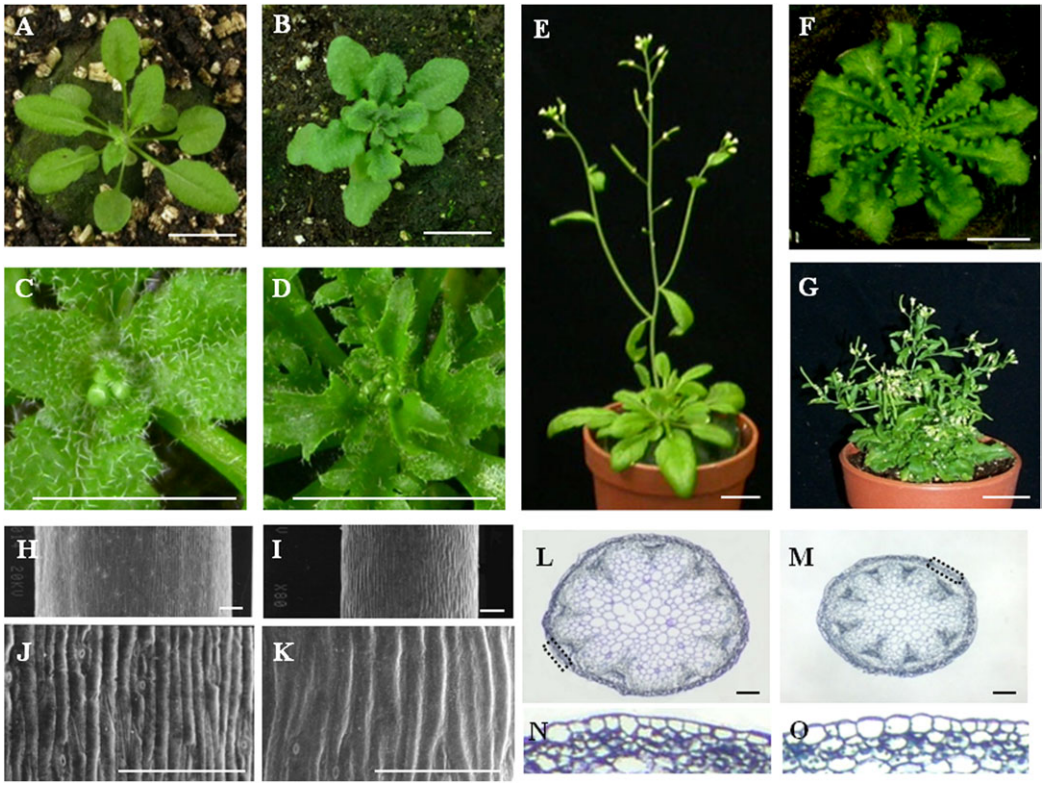

Fig. 4. Phenotypic characterization of *eve1-D/+* plants. (A) A 20-day-old wild-type plant. (B) A 20-day-old *eve1-D/+* plant. (C) Close-up of the shoot of a 20-day-old wild-type plant. (D) Close-up of the shoot of a 20-day-old *eve1-D/+* plant. (E) A 30-day-old wild-type plant. (F) A 30-day-old *eve1-D/+* plant. (G) A 40-day-old *eve1-D/+* plant. (H–K) Scanning electron microscopy images of stems in wild-type (H) and *eve1-D/+* (I) plants; pictures in the same panel of wild-type (J) and *eve1-D/+* (K) plants were taken with the same magnification. (L–O) Toluidin blue-stained cross-section of stems in wild-type (L) and *eve1-D/+* (M) plants; close-up of the epidermis of wild-type (N) and *eve1-D/+* (O) plants, respectively. Bars=100mm in A–G and 100μm in H–M.

was not changed in *eve1-D* plants. In addition, because members of the *YABBY* gene family act redundantly to specify the abaxial identity, transcript levels of the *YABBY* genes, *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*), were examined in *eve1-D* plants. No significant differences in the levels of transcripts of these genes were observed in the *eve1-D* plants compared with the wild-type plants (Fig. 8C). The transcript levels of *PHABULOSA* (*PHB*), which regulates the adaxial polarity cell fate, were slightly increased in *eve1-D* seedlings (Fig. 8C). *APETALA1* (*AP1*) plays an important role in the phase transition (Benlloch *et al.*, 2007). Thus, the expression of the *AP1* gene and the other homeotic genes, *AP2* and *AP3*, in the *eve1-D* plants was also examined. *AP1* and *AP2* expression was significantly down-regulated in the *eve1-D* plants (Fig. 8D). In regard to interaction with KNOX proteins, KNAT1/BP and STM, the expression of *BEL1*-like homeobox genes was examined: *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*), *PENNYWISE* (*PNY*), and *POUNDFOOLISH* (*PNF*) which are necessary for internode patterning and SAM maintenance (Kanrar *et al.*, 2006; Rutjens *et al.*, 2009); and *SAWTOOTH1* (*SAW1*) and *SAW2* which are related to leaf morphology (Kumar *et al.*, 2007). As shown in Fig 8E, the expression of these genes did not show any significant changes.

Discussion

During the vegetative phase of development of *Arabidopsis*, the SAM undergoes a phase transition to become an IM, and the emergence of initial flower buds is followed by formation of the primary inflorescence stem. Much of the current understanding of phase transition from the vegetative to the reproductive state has been gained by examining the regulation of genes related to floral induction and repression in *Arabidopsis*. In practice, a number of genes during this phase transition have been cloned and analysed for their relationship to various aspects of these floral integration pathways (Bastow and Dean, 2003; Amasino, 2004; Boss *et al.*, 2004). Recently, the process of inflorescence stem formation during the phase transition has been explained in terms of temporal and spatial relationships in formation of the floral part (Pouteau and Albertini, 2009). However, little is known about the mechanism of regulation of bolting during the transition from the vegetative to the reproductive phase of growth.

In this study, screening for mutations related to defective inflorescence stem development was undertaken. A mutation (the *eve1-D* mutation) was identified that results in a dramatic failure of IM formation in phase transition, resulting in arrest of plant development at the vegetative stage. In the early stages of vegetative growth, *eve1-D* plants

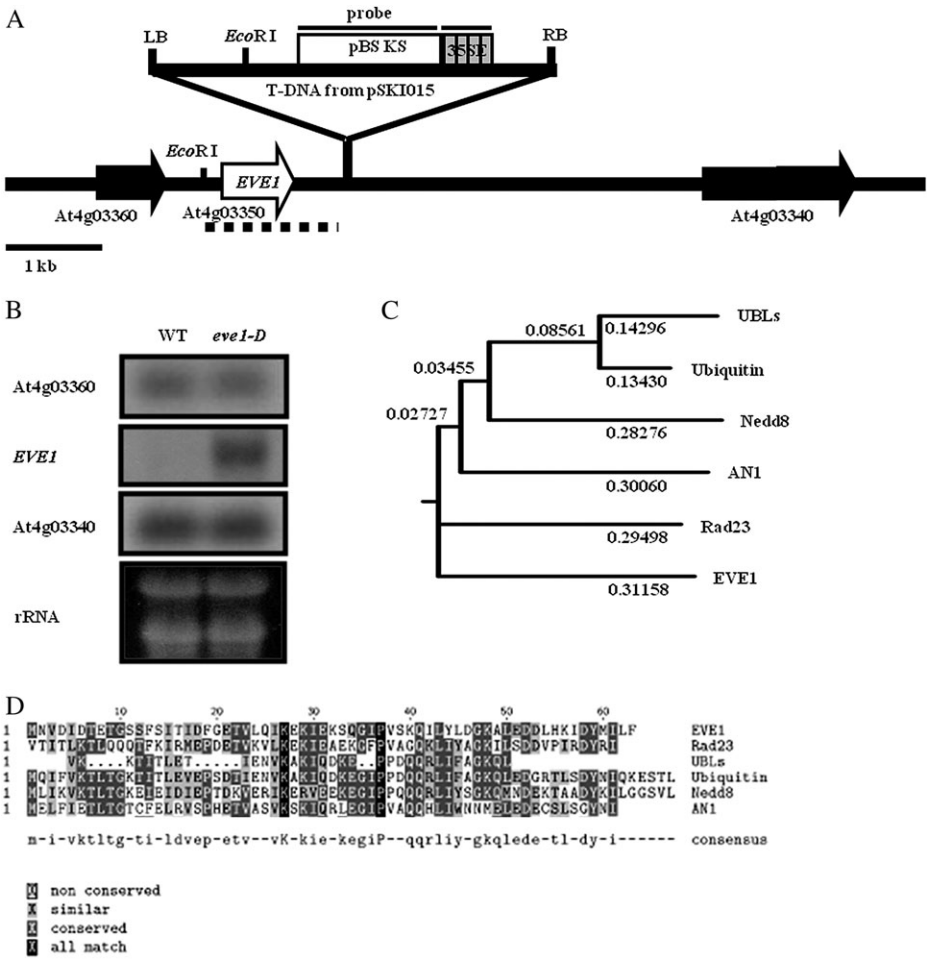

Fig. 5. EVE1, a ubiquitin family protein, is the gene conferring the mutant phenotype. (A) Diagram of the *eve1-D* T-DNA insertion mutant. A T-DNA was inserted in chromosome 4. The right border (RB) and left border (LB) of the T-DNA are indicated by black rectangles. The dotted line represents a sequenced region that was isolated using plasmid rescue. The lines on the pBSK and 35S enhancer represent each probe for Southern hybridization. (B) RNA gel blot analysis of *EVE1* gene expression in wild-type and *eve1-D* plants. Total RNA was extracted from 14-day-old wild-type and *eve1-D* plants grown on MS plates, and 40mg of total RNA was loaded in each lane. The ethidium bromide staining pattern of rRNAs shows equal loading. (C) Phylogenetic tree based on the amino acid sequences. Numbers above branches are genetic distances based on gap open penalty (10.00). The tree was obtained using the Phylip-format dendrogram from Workbench. UBLs (Ubiquitin-like domain, Q15011), ubiquitin (P23324), Nedd8 (NP_609919), AN1 (NP_777550), and Rad23 (T04150). (D) Multiple sequence alignment of the ubiquitin domain of EVE1 and ubiquitin superfamily proteins from *Arabidopsis* using CLUSTALW (http://workbench.sdsc.edu). A black background indicates 100% conservation, dark grey is 80%, and light grey is 60%.

produced leaf primordia at the flanks of the normal dome-shaped SAM. During the period when wild-type plants undergo phase transition from vegetative growth to the reproductive phase of development, the vegetative SAM of the *eve1-D* mutant did not transition to IM. The *eve1-D* mutant showed axillary and lateral SAMs in the late vegetative stage but it could not generate axillary and lateral inflorescences. The defective SAM or no-inflorescence phenotypes are similar to those seen in some other mutants such as *stm* and *sha1*. The *stm* mutant exhibited a defective SAM and did not generate rosette leaves. STM is required for SAM formation during embryogenesis (Long *et al.*, 1996). The regulation of SAM maintenance is reported to involve SHA1, a C4HC3-type RING finger protein. The *sha1* mutant exhibited a defective SAM that could not elongate into the initial primary inflorescence stem. Ectopic meristems were formed around the terminated SAM at later growth stages and produced adventitious shoots and flowers. As compared with these mutants, the overexpression of the *EVE1* gene had the novel effect of completely suppressing the formation of the primary, axillary, and lateral inflorescence stem during phase transition from the vegetative to the reproductive phase.

A large number of genes related to SAM identity, SAM maintenance, leaf morphology, and floral integrators have been reported to be involved in SAM development as well as the phase transition. To determine the relationship of these genes to the *eve1-D* mutation, the expression levels of a number of these genes were analysed in *eve1-D* mutant plants. Only the transcript levels of the meristem identity genes, *AP1* and *AP2*, exhibited significant changes in expression in the *eve1-D* plants. The *AP1* and *AP2*

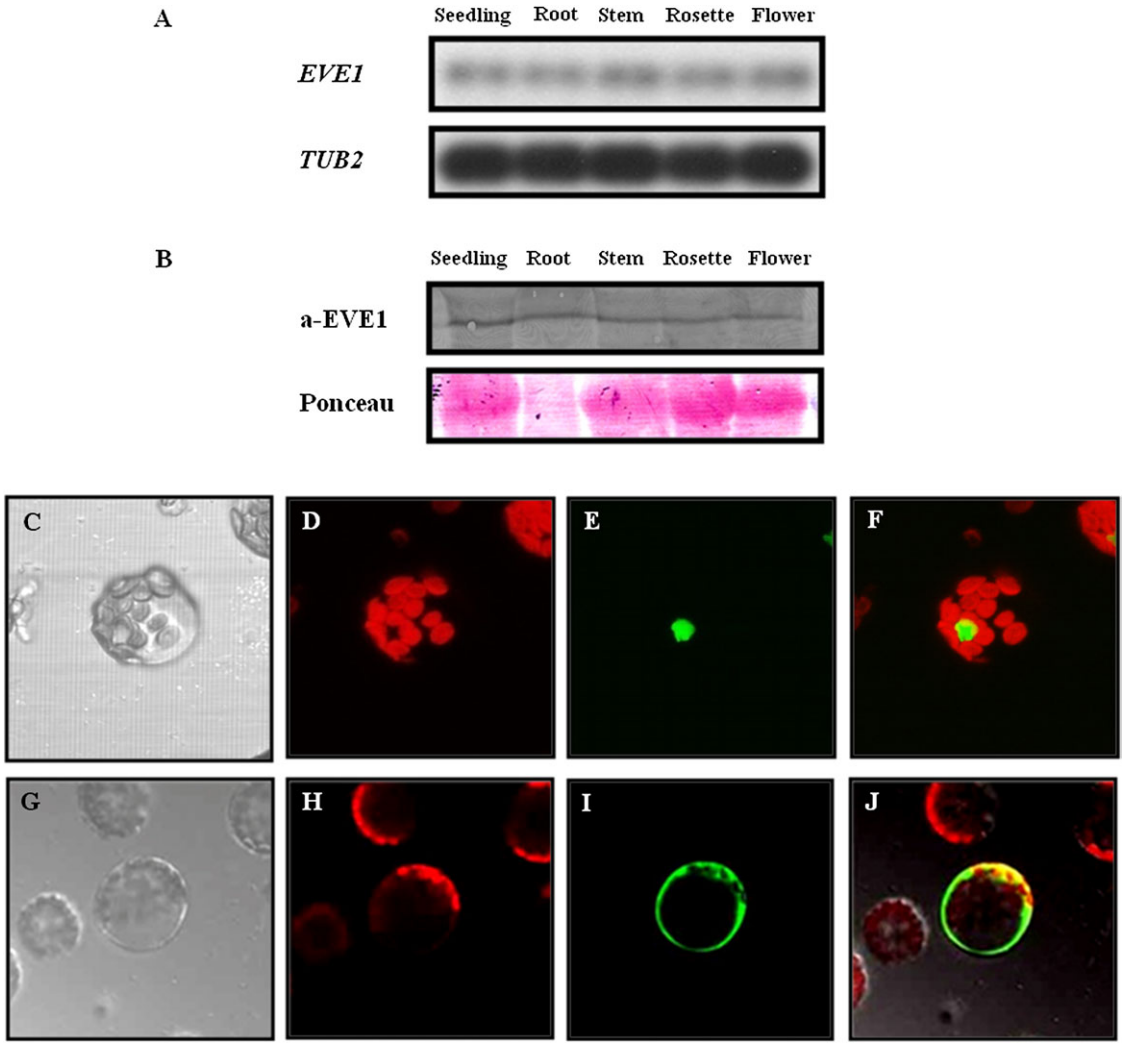

Fig. 6. Expression pattern analysis. (A) RT-PCR analysis of *EVE1* gene expression in different tissues of wild-type plants. The number of cycles was 28 for *EVE1* (top) and 24 for *TUB2* (bottom). *TUB2* (*β-tubulin 2*) was used as control. The RT-PCR product of *EVE1* was detected by DNA gel blot analysis using ^{32}P-labelled probes because of their low expression level. (B) Western blot analysis of EVE1 protein expression in various organs of *Arabidopsis*. (C–E and G–J) Nuclear localization of EVE1–GFP in *Arabidopsis* leaf protoplast. Chloroplasts appear red (pseudo colour). GFP is green. (C, G) Transparent images of protoplasts. (D, H) Chloroplast autofluorescence. (E, I) EVE1–GFP and 35S:GFP fluorescence. (F) Merged image of EVE1–GFP and chlorophyll fluorescence. (J) Images of 35S:GFP and chloroplast fluorescence were merged. 35S:GFP was used as a control.

genes encode the floral homeotic genes and play a role in determinate development of the floral meristem (Irish and Sussex, 1990). *AP1* regulates the promotion of floral organ formation, or inflorescence commitment (Ng and Yanofsky, 2001). During phase transition, the vegetative meristem is initially converted into the inflorescence meristem, which then produces floral meristems on its flanks of the SAM. The regulation of floral transition is controlled by the floral meristem identity gene, *AP1* (Komeda, 2004; Blazquez, 2005). Axillary meristems acquire a floral identity primarily through the activity of the meristem identity genes *LFY* and *AP1* (Liljegren *et al.*, 1999). *AP2* is involved in the various developmental processes at the shoot apex, including the regulation of the stem cell niche and floral organ determination (Bowman *et al.*, 1989; Wurschum *et al.*, 2006). Recently, the dual function of *AP2* has been explained as a stimulator and a repressor in floral transition and floral development (Yant *et al.*, 2010). Combined with these data, the results demonstrate that *EVE1* controls the inflorescence stem development related to *AP1*/*AP2* regulation.

The EVE1 protein is a ubiquitin family protein that contains the ubiquitin domain. The ubiquitin family proteins are involved in many aspects of DNA repair, embryogenesis, transcriptional regulation, and apoptosis (Vandenberg *et al.*, 2003; Zhang *et al.*, 2008; Xu *et al.*, 2009). Recently, it has been reported that the C4HC3-type RING finger protein containing ubiquitin protein E3 ligase (SHA1) arrests the primary inflorescence in the WUS pathway (Sonoda *et al.*, 2007). These data show that ubiquitins and ubiquitin-related proteins play important roles in the regulation of *Arabidopsis* development.

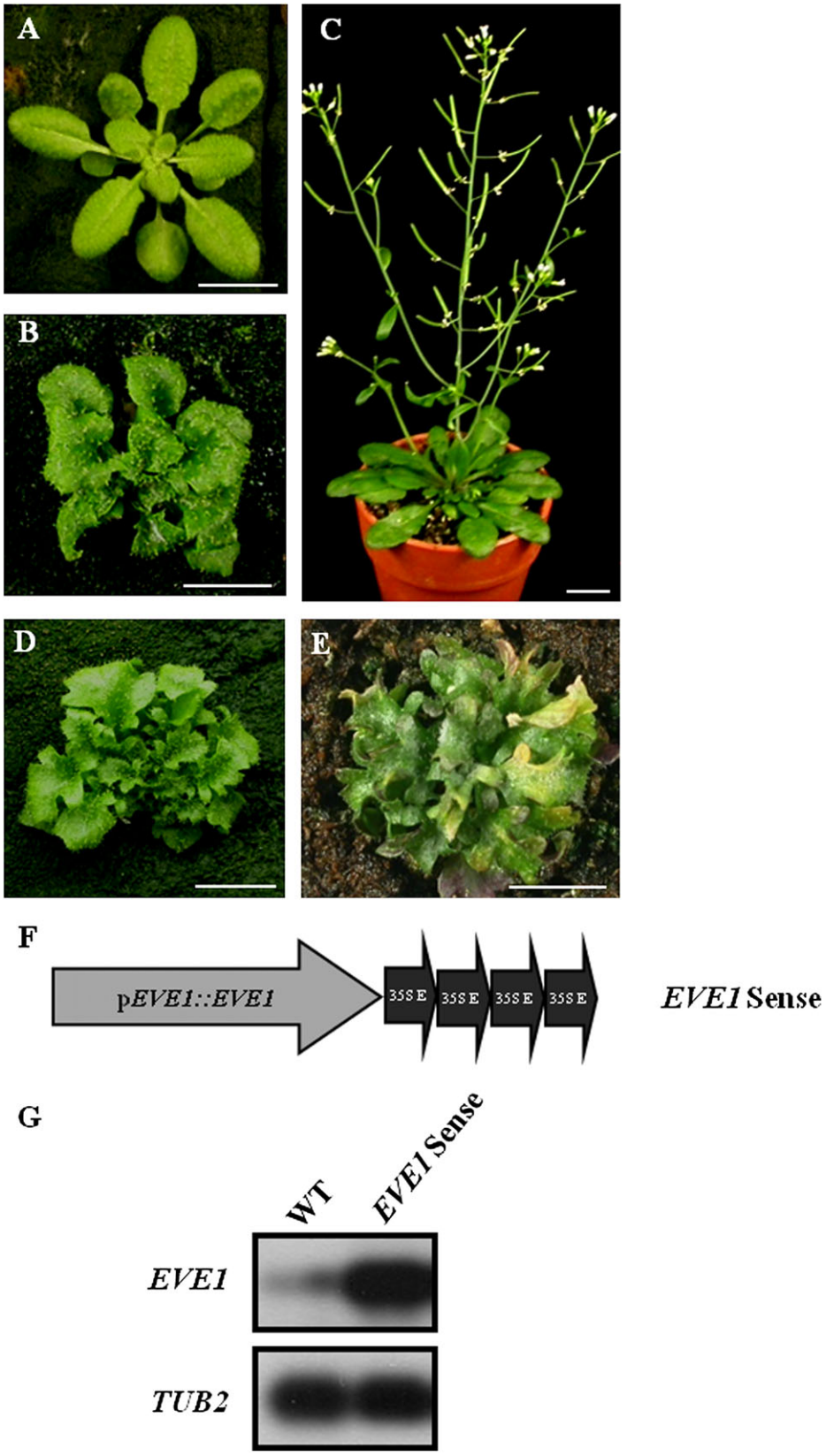

Fig. 7. Phenotypic and molecular characterization of *EVE1* transgenic plants. (A, B) Phenotypic comparison of sense transformants with a 20-day-old wild-type plant (A) and an *EVE1*-overexpressing line (B). (C, D) A 35-day-old wild type plant (C) and an *EVE1*-overexpressing plant (D). (E) A 45-day-old *EVE1*-overexpressing plant. (F) Schematic structure of the *EVE1* sense construct. (G) RT-PCR analysis of the *EVE1* expression level in wild-type plants and *EVE1*-overexpressing plants. The RT-PCR product of *EVE1* was detected by DNA gel blot analysis using ^{32}P-labelled probes because of their low expression level. *TUB2* (*β-tubulin 2*) was used as a control. Bars=100mm.

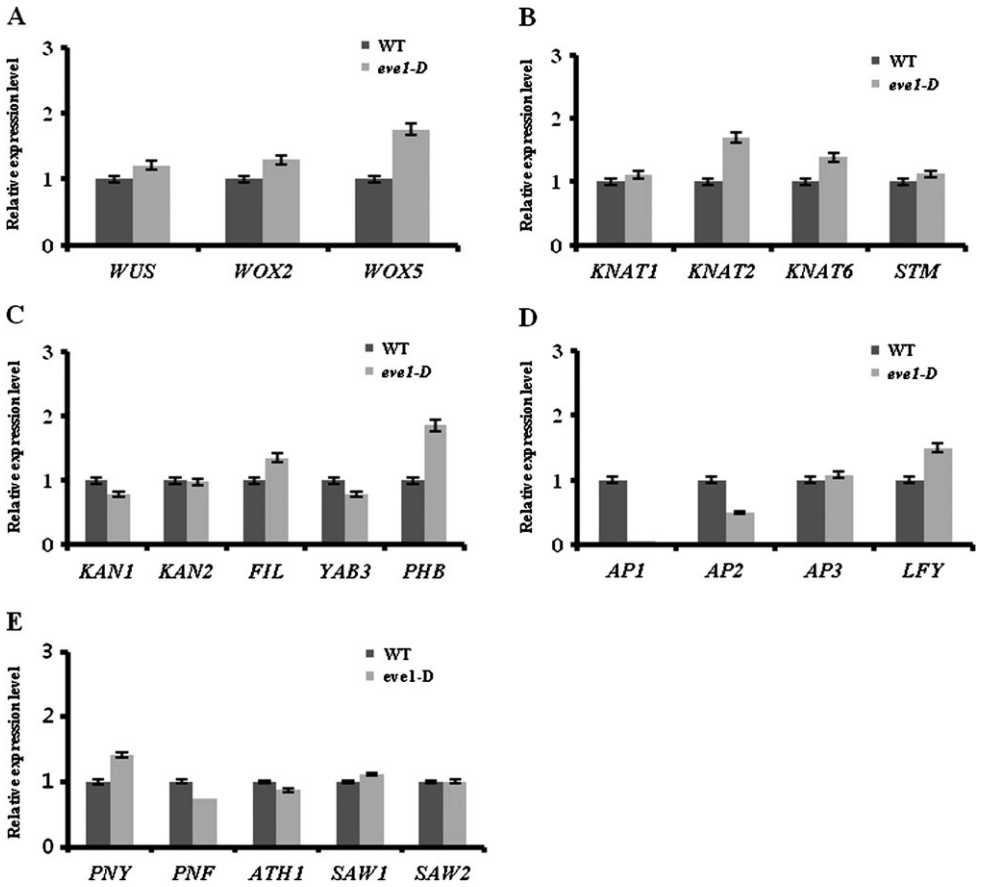

Fig. 8. Real-time PCR analyses of various genes in 14-day-old wild-type and *eve1-D* plants. (A) Expression of *WUS*, *WOX2*, and *WOX5*. (B) Expression of *KNAT1*, *KNAT2*, *KNAT6*, and *STM*. (C) Expression of *KAN1*, *KAN2*, *FIL*, *YAB3*, and *PHB*. (D) Expression of *AP1*, *AP2*, *AP3*, and *LFY*. (E) Expression of *PNY*, *PNF*, *ATH1*, *SAW1*, and *SAW2*. Expression levels were normalized to *β-tubulin 2* (*TUB2*) gene expression. Black bars represent wild-type seedlings (left), and grey bars represent *eve1-D* seedlings (right). The values are given as means±SD, n=5.

The function of ubiquitin family proteins in relation to inflorescence development and phase transition is still unknown in higher plants. In this report, the fact that overexpression of the *EVE1* gene alters leaf, shoot, and fruit development may suggest that EVE1 regulates growth during inflorescence stem development and may be particularly involved in the establishment of the *Arabidopsis* indeterminate inflorescence. Therefore, further analysis of this mutation will help us to understand the mechanism controlling phase transition in *Arabidopsis*.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Morphology of *eve1-D*/+ plants.

Figure S2. Analysis of transgenic *Arabidopsis* plants expressing antisense *EVE1* mRNA.

Table S1. Morphological analysis of wild-type and *eve1-D*/+ plants.

Table S2. Primers used in real-time PCR.

Acknowledgements

We wish to thank Professor Dr Donald J. Amstrong, Oregon State University, Corvallis, OR, USA for his critical review of this manuscript. This work was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-314-C00275 and KRF-2008-313-C00841) and by a BK21 Research Fellowship from the Ministry of Education, Science and Technology, Republic of Korea.

References

Amasino R. 2004. Vernalization, competence, and the epigenetic memory of winter. *The Plant Cell* **16,** 2553–2559.

Bastow R, Dean C. 2003. Plant sciences. Deciding when to flower. *Science* **302,** 1695–1696.

Baurle I, Dean C. 2006. The timing of developmental transitions in plants. *Cell* **125,** 655–664.

Benlloch R, Berbel A, Serrano-Mislata A, Madueno F. 2007. Floral initiation and inflorescence architecture: a comparative view. *Annals of Botany* **100,** 659–676.

Blazquez MA. 2005. Plant science. The right time and place for making flowers. *Science* **309,** 1024–1025.

Boss PK, Bastow RM, Mylne JS, Dean C. 2004. Multiple pathways in the decision to flower: enabling, promoting, and resetting. *The Plant Cell* **16 Suppl** S18–S31.

Bowman JL, Smyth DR, Meyerowitz EM. 1989. Genes directing flower development in Arabidopsis. *The Plant Cell* **1,** 37–52.

Chuck G, Lincoln C, Hake S. 1996. KNAT1 induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. *The Plant Cell* **8,** 1277–1289.

Clark SE, Jacobsen SE, Levin JZ, Meyerowitz EM. 1996. The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in Arabidopsis. *Development* **122,** 1567–1575.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *The Plant Journal* **16,** 735–743.

Douglas SJ, Chuck G, Dengler RE, Pelecanda L, Riggs CD. 2002. KNAT1 and ERECTA regulate inflorescence architecture in Arabidopsis. *The Plant Cell* **14,** 547–558.

Endrizzi K, Moussian B, Haecker A, Levin JZ, Laux T. 1996. The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. *The Plant Journal* **10,** 967–979.

Hofmann RM, Pickart CM. 2001. *In vitro* assembly and recognition of Lys-63 polyubiquitin chains. *Journal of Biological Chemistry* **276,** 27936–27943.

Irish VF, Sussex IM. 1990. Function of the apetala-1 gene during Arabidopsis floral development. *The Plant Cell* **2,** 741–753.

Kanrar S, Onguka O, Smith HM. 2006. Arabidopsis inflorescence architecture requires the activities of KNOX–BELL homeodomain heterodimers. *Planta* **224,** 1163–1173.

Komeda Y. 2004. Genetic regulation of time to flower in Arabidopsis thaliana. *Annual Review of Plant Biology* **55,** 521–535.

Kumar R, Kushalappa K, Godt D, Pidkowich MS, Pastorelli S, Hepworth SR, Haughn GW. 2007. The Arabidopsis BEL1-LIKE HOMEODOMAIN proteins SAW1 and SAW2 act redundantly to regulate KNOX expression spatially in leaf margins. *The Plant Cell* **19,** 2719–2735.

Lee SY, Kim H, Hwang HJ, Jeong YM, Na SH, Woo JC, Kim SG. 2010. Identification of tyrosyl-DNA phosphodiesterase as a novel DNA damage repair enzyme in Arabidopsis. *Plant Physiology* **154,** 1460–1469.

Lenhard M, Jurgens G, Laux T. 2002. The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in Arabidopsis shoot meristem regulation. *Development* **129,** 3195–3206.

Liljegren SJ, Gustafson-Brown C, Pinyopich A, Ditta GS, Yanofsky MF. 1999. Interactions among APETALA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. *The Plant Cell* **11,** 1007–1018.

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S. 1994. A knotted1-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *The Plant Cell* **6,** 1859–1876.

Long JA, Moan EI, Medford JI, Barton MK. 1996. A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature* **379,** 66–69.

Medford JI, Behringer FJ, Callos JD, Feldmann KA. 1992. Normal and abnormal development in the Arabidopsis vegetative shoot apex. *The Plant Cell* **4,** 631–643.

Ng M, Yanofsky MF. 2001. Activation of the Arabidopsis B class homeotic genes by APETALA1. *The Plant Cell* **13,** 739–753.

Pouteau S, Albertini C. 2009. The significance of bolting and floral transitions as indicators of reproductive phase change in Arabidopsis. *Journal of Experimental Botany* **60,** 3367–3377.

Reddy GV, Meyerowitz EM. 2005. Stem-cell homeostasis and growth dynamics can be uncoupled in the Arabidopsis shoot apex. *Science* **310,** 663–667.

Rutjens B, Bao D, van Eck-Stouten E, Brand M, Smeekens S, Proveniers M. 2009. Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins. *The Plant Journal* **58,** 641–654.

Sonoda Y, Yao SG, Sako K, Sato T, Kato W, Ohto MA, Ichikawa T, Matsui M, Yamaguchi J, Ikeda A. 2007. SHA1, a novel RING finger protein, functions in shoot apical meristem maintenance in Arabidopsis. *The Plant Journal* **50,** 586–596.

Vandenberg CJ, Gergely F, Ong CY, Pace P, Mallery DL, Hiom K, Patel KJ. 2003. BRCA1-independent ubiquitination of FANCD2. *Molecular Cell* **12,** 247–254.

Venglat SP, Dumonceaux T, Rozwadowski K, Parnell L, Babic V, Keller W, Martienssen R, Selvaraj G, Datla R. 2002. The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **99,** 4730–4735.

Weigel D, Ahn JH, Blazquez MA, *et al*. 2000. Activation tagging in Arabidopsis. *Plant Physiology* **122,** 1003–1013.

Wurschum T, Gross-Hardt R, Laux T. 2006. APETALA2 regulates the stem cell niche in the Arabidopsis shoot meristem. *The Plant Cell* **18,** 295–307.

Xu L, Menard R, Berr A, Fuchs J, Cognat V, Meyer D, Shen WH. 2009. The E2 ubiquitin-conjugating enzymes, AtUBC1 and AtUBC2, play redundant roles and are involved in activation of FLC expression and repression of flowering in Arabidopsis thaliana. *The Plant Journal* **57,** 279–288.

Yant L, Mathieu J, Dinh TT, Ott F, Lanz C, Wollmann H, Chen X, Schmid M. 2010. Orchestration of the floral transition and floral development in Arabidopsis by the bifunctional transcription factor APETALA2. *The Plant Cell* **22,** 2156–2170.

Zhang Y, Feng S, Chen F, Chen H, Wang J, McCall C, Xiong Y, Deng XW. 2008. Arabidopsis DDB1-CUL4 ASSOCIATED FACTOR1 forms a nuclear E3 ubiquitin ligase with DDB1 and CUL4 that is involved in multiple plant developmental processes. *The Plant Cell* **20,** 1437–1455.