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

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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 SHAI 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 overexpression 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 pSki105 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 EcoRI-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′-AAGG-TACGTTTGATCCTAATCG-3′ and 5′-AACTGCAGCT-

CACCCTCATGGAT-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 PstI and SalI and ligated into the PstI and SalI sites of pMN20 for complementation. For transgenic antisense lines, the primers used to generate the EVE1 ORF were 5′-GGGAATACAGCTTGTGATCACTA-3′ and 5′-AACAGTTTCAAGCTGAG-3′. The PCR product was digested with BamHI and ligated into the BamHI sites of the binary vector pBI121 in antisense orientation. Transgenic plants were generated in the wild type by floral dipping and selected by 50mg 1−1 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 (ACT) 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 S1 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′-AAAGATCTCAAATGAACGGACGTC-3′) and G-R (5′-TTGATCTTACCTTCAGCGATA-3′) primers containing a BamHI 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

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 (ACT) 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

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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).

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 auxiliary 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

![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.](https://academic.oup.com/jxb/article-abstract/62/13/4571/490813)
The stem width critically decreased in eve1-D plants (Supplementary Table S1) than those of wild-type plants (Fig. 2B). Rosette leaves of a 25-day-old wild-type 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) A 25-day-old eve1-D plant. (J) Magnified SAM of a 25-day-old eve1-D plant. (L) 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 wild-type plant. (M) A 25-day-old eve1-D plant. (N) Magnified SAM of 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.

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, Ned8, 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
A 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 \(WUS\), \(WUSCHEL RELATED HOMEBOX 2\) (\(WOX2\)), 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 \(eve1-D\) plants (Fig. 8B). In relation to leaf polarization, the expression of \(KANADI1\) (\(KANI\)) and \(KAN2\) genes was analysed and it was found that the expression of these genes...
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 HOMEODOMAIN 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...
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...
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. 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 32P-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.
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 32P-labelled probes because of their low expression level. TUB2 (β-tubulin 2) was used as a control. Bars=100mm.
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 \textit{EVE1} gene alters leaf, shoot, and fruit development may suggest that \textit{EVE1} regulates growth during inflorescence stem development and may be particularly involved in the establishment of the \textit{Arabidopsis} indeterminate inflorescence. Therefore, further analysis of this mutation will help us to understand the mechanism controlling phase transition in \textit{Arabidopsis}.

**Supplementary data**

Supplementary data are available at \textit{JXB} online.

- Figure S1. Morphology of \textit{eve1}-D/+ plants.
- Figure S2. Analysis of transgenic \textit{Arabidopsis} plants expressing antisense \textit{EVE1} mRNA.
- Table S1. Morphological analysis of wild-type and \textit{eve1}-D/+ plants.
- Table S2. Primers used in real-time PCR.

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