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RESEARCH PAPER

Convergence of the 26S proteasome and the *REVOLUTA* pathways in regulating inflorescence and floral meristem functions in *Arabidopsis*

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Abstract

The 26S proteasome is a large multisubunit proteolytic complex, regulating growth and development in eukaryotes by selective removal of short-lived regulatory proteins. Here, it is shown that the 26S proteasome and the transcription factor gene *REVOLUTA* (*REV*) act together in maintaining inflorescence and floral meristem (IM and FM) functions. The characterization of a newly identified *Arabidopsis* mutant, designated *ae4* (*asymmetric leaves1/2 enhancer4*), which carries a mutation in the gene encoding the 26S proteasome subunit, RPN2a, is reported. *ae4* and *rev* have minor defects in phyllotaxy structure and meristem initiation, respectively, whereas *ae4 rev* demonstrated strong developmental defects. Compared with the *rev* single mutant, an increased percentage of *ae4 rev* plants exhibited abnormal vegetative shoot apical and axillary meristems. After flowering, *ae4 rev* first gave rise to a few normal-looking flowers, and then flowers with reduced numbers of all types of floral organs. In late reproductive development, instead of flowers, the *ae4 rev* IM produced numerous filamentous structures, which contained cells seen only in the floral organs, and then carpelloid organs. *In situ* hybridization revealed that expression of the *WUSCHEL* and *CLAVATA3* genes was severely down-regulated or absent in the late appearing *ae4 rev* primordia, but the genes were strongly expressed in top-layer cells of inflorescence tips. Double mutant plants combining *rev* with other 26S proteasome subunit mutants, *rpn1a* and *rpn9a*, resembled *ae4 rev*, suggesting that the 26S proteasome might act as a whole in regulating IM and FM functions.

Key words: *Arabidopsis*, floral meristem, inflorescence meristem, 26S proteasome, *REVOLUTA*.

Introduction

The plant shoot apical meristem (SAM) is responsible for generating all above-ground organs, including leaves, stems, and flowers. In flowering plants, the SAM undergoes several distinct transitions in identity during the life of the plant (Pidkowich *et al.*, 1999). One important transition is the switch from vegetative growth to reproductive growth, resulting in the conversion of a vegetative meristem to an inflorescence meristem (IM). In *Arabidopsis*, the IM subsequently produces floral meristems (FMs), which give rise to four types of floral organs: four sepals, four petals, six stamens, and two fused carpels (Bowman *et al.*, 1989). The SAM tissue is established during embryogenesis, when *WUSCHEL-CLAVATA* (*WUS-CLV*) and *SHOOT MERISTEMLESS* (*STM*) pathways are activated (Clark *et al.*, 1996; Lenhard *et al.*, 2002). *WUS* encodes a nuclear-localized homeodomain protein and is expressed in the organizing centre domain underneath the shoot stem cells. The *wus* mutant prematurely terminates its SAM, due to a failure to maintain sufficient numbers of stem cells (Laux *et al.*, 1996; Mayer *et al.*, 1998; Lenhard *et al.*, 2002).

In the SAM, the number of cells expressing *WUS* is controlled by three *CLV* genes, namely *CLV1*, *2*, and *3*, which encode a leucine-rich repeat (LRR)-receptor kinase, an LRR receptor-like protein, and a small secreted peptide,

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respectively (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Jeong *et al.*, 1999). The *clv1*, *2*, and *3* mutants displayed similar phenotypes, showing enlarged vegetative SAM, IM, and FM with overproliferated stem cells. In contrast, over-expression of *CLV3* resulted in *wus*-like plants, in which the *WUS* gene was severely repressed (Brand *et al.*, 2000). It was proposed that the CLV3 peptide is secreted from stem cells and acts as a signalling molecule that binds to and activates the CLV1/CLV2 receptor complex (Fletcher *et al.*, 1999; Trotochaud *et al.*, 1999; Rojo *et al.*, 2002). Signalling from the *CLV* pathway limits *WUS* expression in a specified region; and the *CLV3* expression, in turn, is induced by *WUS*. Therefore, a feedback loop is formed to control the number of stem cells dynamically and precisely, and maintain the integrity of a normal meristem (Brand *et al.*, 2000; Schoof *et al.*, 2000; Muller *et al.*, 2006). The *WUS-CLV* pathways also maintain stem cell numbers in the FM until the specification of the floral organ carpels (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001).

Genes in the class III homeodomain-leucine zipper (HD-ZIP III) family are also important for meristem formation and maintenance (Talbert *et al.*, 1995; Otsuga *et al.*, 2001; Emery *et al.*, 2003; Prigge *et al.*, 2005). It was reported that *REVOLUTA* (*REV*), one of the HD-ZIP III genes, plays roles in initiation of both axillary meristem (AM) and FM, as AMs frequently failed to develop in both rosette and cauline leaf axils, and flowers lacked full meristematic activity in the loss-of-function *rev* mutants (Talbert *et al.*, 1995; Otsuga *et al.*, 2001). Two additional genes in this family, *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), perform overlapping functions with *REV*, as *phb* and *phv* both appeared normal, whereas *rev phb* and *rev phb phv* initiated little post-embryonic organ growth (Emery *et al.*, 2003; Prigge *et al.*, 2005).

The 26S proteasome, a proteolytic complex containing 31 subunits, is essential to fundamental plant biology.4 Previous studies showed that mutations in the single subunit genes in *Arabidopsis* could result in plants with abnormal root and shoot meristems (Ueda *et al.*, 2004), defective leaf polarity establishment (Huang *et al.*, 2006), and aberrant cell division (Kurepa *et al.*, 2009). In this study, the characterization of a weak 26S proteasome subunit mutant, *ae4*/*rpn2a*, is reported. By analysing an *ae4 rev* double mutant, a new role for both the 26S proteasome and *REV* genes in regulating plant meristem activities was revealed. Apart for their roles in initiation and maintenance of plant meristems as previously reported (Otsuga *et al.*, 2001; Ueda *et al.*, 2004; Prigge *et al.*, 2005), both the proteasome and *REV* pathways are required for regulating IM and FM functions, and this regulation, at least partially, involves the *WUS-CLV* network.

Materials and methods

Plant materials and growth conditions

Identification of the *as2-101* mutant (Landsberg *erecta*, L*er*) and mutant screening for *as2* enhancers were described in previous work (Sun *et al.*, 2000; Li *et al.*, 2005). The newly obtained *as2* enhancer mutant was backcrossed to wild-type L*er* three times before detailed phenotypic analyses. The *rev-6* and *rev-9* alleles, both in the L*er* background, were kindly provided by S. E. Clark and J. L. Bowman, respectively. T-DNA insertion mutants used in this work are all in the Col-0 background, among which *rpn1a-2* (SALK_129604) and *rpn9a-1* (SALK_147710) were previously studied (Huang *et al.*, 2006), and *rpn2a* (SALK_133787, also called *ae4-2* in this work) was newly obtained from the ABRC. Genotyping of *ae4-2* was performed using primers 5′-TAGTGTTCTCCATCAATGG-3′ and 5′-CTTAGAGACCA-GCAAAGC-3′ plus a T-DNA left border primer 5′-TGGTTCA-CGTAGTGGGCCATCG-3′. Reverse transcription-PCR (RT-PCR) was further carried out to verify the loss-of-function mutation in *ae4-2* (Supplementary Fig. S1 available at *JXB* online). Plants were grown according to previous conditions (Chen *et al.*, 2000).

Complementation analysis of *ae4-1* was performed as follows: a fragment containing the *RPN2a* coding sequence was first generated by RT-PCR, using cDNAs made from leaves of wild-type L*er*; after sequencing verification, this fragment was inserted into a modified *pCAMBIA2300* transformation vector under the control of the cauliflower mosaic virus 35S promoter, and the resultant construct carried by the *Agrobacterium* strain GV3101 was introduced into the *ae4-1 rev-6* double mutant plants by the floral-dip method.

In situ *hybridization*

In situ hybridization was performed according to a previously described method (Long *et al.*, 1996). The *WUS* and *CLV3* probes were made from constructs containing cDNA fragments, yielded by RT-PCR using the following primers: 5′-TAACAAGCCA-TATCCCAGC-3′ and 5′-GCTTTAATCCCGAGCGAC-3′ for *WUS*; and 5′-TTCACCAGATCTCACTCAAG-3′ and 5′-CTTA-CATTCACTTCAGCAAC-3′ for *CLV3*. These cDNA fragments were subcloned into pBluescript SK for sequencing verification. The digoxigenin-labelled RNA probes were generated using the DIG RNA labelling kit according to the manufacturer's instruction (Roche, Branchburg, NJ, USA).

Microscopy

Fresh tissues from wild-type and mutant plants were examined using a SZH10 dissecting microscope (Olympus, Tokyo, Japan) and photographed using a Nikon E995 digital camera (Nikon, Tokyo, Japan). The scanning electron microscopy observation and preparation of specimens were according to previous methods (Chen *et al.*, 2000).

Results

Characterization of the ae4-1 *mutant and identification of the* AE4 *gene*

During a continuing effort to identify factors in the *Arabidopsis ASYMMETRIC LEAVES1/2* (*AS1* and *AS2*) regulatory network, a single *as2-101* enhancer mutant, designated as *ae4-1* (*asymmetric leaves1/2 enhancer4*), was isolated by ethylmethane sulphonate (EMS) mutagenesis of *as2-101* and mutant screening. The *ae4-1* single mutant (Fig. 1C) exhibited no obvious developmental defects at seedling stages compared with the wild type (Fig. 1A), whereas *as2-101* only showed weak leaf polarity defects (Fig. 1B) (Xu *et al.*, 2002, 2003). However, the double mutant *ae4-1 as2-101* apparently enhanced the leaf polarity defects of *as2-101*. Notably, almost all the *ae4-1 as2-101* plants produced abaxialized lotus leaves, in which the leaf petiole was

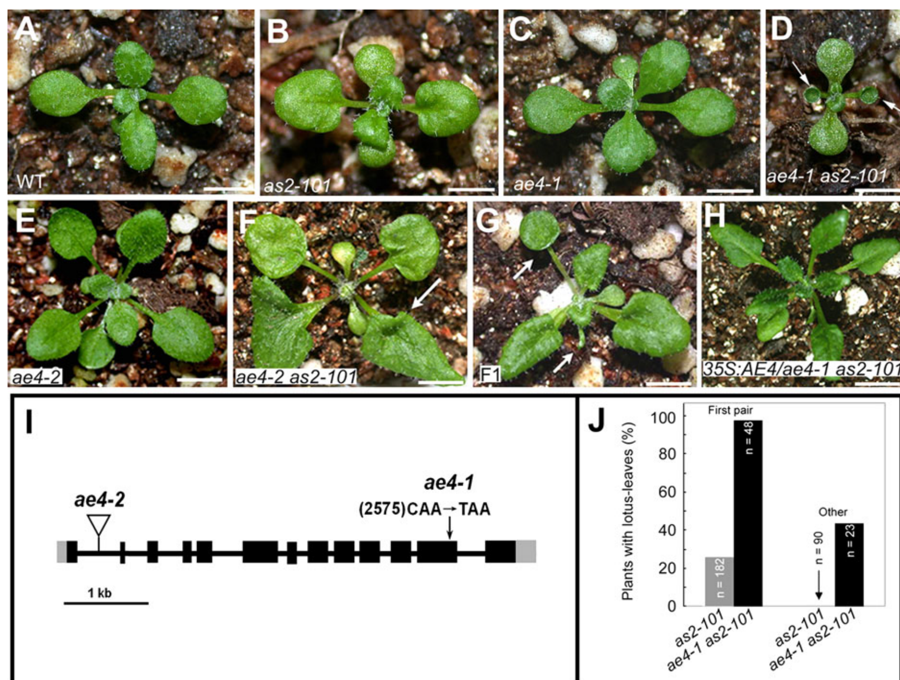

Fig. 1. *ae4* enhanced the leaf polarity defects of *as2*. (A–F) Morphological observations of the wild-type L*er* (A), and the *ae4-1* (B), *as2-101* (C), *ae4-1 as2-101* (D), *ae4-2* (E), and *ae4-2 as2-101* (F) mutants. (G) Seedlings of F_1 progeny of a cross between *ae4-1 as2-101* and *ae4-2 as2-101* showed the enhanced *as2* leaf phenotypes with increased lotus- and needle-like structures. (H) An *ae4-1 as2-101* plant transformed with *35S:AE4* exhibited only *as2* single mutant phenotypes. Arrows in (D), (F), and (G) indicate the abaxialized lotus- and needle-like leaves. (I) A diagram shows the *AE4* (*RPN2a*) gene structure with the *ae4-1* and *ae4-2* mutations. Black and grey boxes show the exons and untranslated regions, respectively, and horizontal lines indicate introns. (J) Compared with the *as2-101* single mutant, *ae4-1 as2-101* displayed increased polarity-defective leaves. n, numbers of plants analysed; first pair, the first pair of rosette leaves; other, other rosette leaves. Bars=0.5 cm in A–H.

attached underneath the lamina (Fig. 1D, arrows). In contrast, only a small percentage of such leaves were observed in *as2-101* plants (Fig. 1J). In addition, the lotus leaves in *as2-101* were found only among the first two appearing rosette leaves, whereas *ae4-1 as2-101* plants produced such structures in rosette leaves that appeared later (Fig. 1J).

To identify the *AE4* gene, ~2700 recombinants were analysed, and the *AE4* gene was mapped to the lower arm of chromosome 2, within a 190 kb region. It has previously been reported that mutations in a number of 26S proteasome subunit genes resulted in severely abaxialized rosette leaves when combined with the *as2-101* mutation (Huang *et al.*, 2006). As the 190 kb region contained a gene (At2g32730) encoding a putative 26S proteasome subunit RPN2a, this gene was the first to have its sequence analysed. A C-to-T substitution in the 12th exon was identified that led to a premature stop codon in the *RPN2a* gene (Fig. 1I).

To determine whether the defective *RPN2a* gene is responsible for the enhanced leaf phenotypes in the *as2-101* enhancer mutant, an allelism test was first performed using an additional *rpn2a* allele (SALK_133787, now called *ae4-2*) (Fig. 1E), which has a T-DNA insertion in the first intron of the *RPN2a* gene (Fig. 1I). Like *ae4-1*, the double mutant formed by combining *ae4-2* with *as2-101* produced increased numbers of lotus leaves (Fig. 1F) compared with the *as2-101* single mutant. In addition, the F_1 progeny from eight independent crosses between *ae4-1 as2-101* and *ae4-2 as2-101* all resulted in similarly enhanced *as2-101* phenotypes, showing more lotus leaves or needle-like structures (Fig. 1G). Moreover, the *ae4-1 as2-101* double mutant was transformed with a complementation construct (see Materials and methods). A total of 61 transgenic lines were obtained, of which 60 plants showed only *as2-101* single mutant phenotypes (Fig. 1H). All these results indicate that mutation in the *RPN2a* gene resulted in enhanced *as2-101* phenotypes.

ae4 rev *exhibited SAM and AM defects*

Previous results showed that although *rev* single mutants exhibited weak SAM defects and normal leaf phenotypes, double mutants combining *rev* with the 26S proteasome mutant *ae3*/*rpn8a* resulted in a severely defective vegetative SAM and leaf polarity, with almost all plants arresting at the seedling stages (Huang *et al.*, 2006). These results suggested that the 26S proteasome plays roles in regulating meristematic tissues. However, the *ae3*/*rpn8a rev* phenotypes were very strong, which limited a detailed analysis of the 26S proteasome function in maintaining meristematic tissues.

The newly isolated *ae4-1* appeared to affect the 26S proteasome function mildly, as phenotypes of *ae4-1* are not as strong as those in *ae3*/*rpn8a*. To understand the role of the 26S proteasome in regulating meristem activities, the

ae4-1 rev-6 double mutant was constructed and characterized. Previous results showed that ~70% of the cauline leaves of *rev-6* lacked an AM and ~12% of flowers produced only a limited set of flower organs, preferentially lacking inner organs with the defective FM (Otsuga *et al.*, 2001). However, the *rev-6* phenotypes might be sensitive to environmental conditions, as the phenotypes were much less pronounced under the growth conditions used (see below). Although most *ae4-1 rev-6* seedlings could pass through the vegetative phase (Fig. 2A), the frequency of plants with a defective SAM was much higher in *ae4-1 rev-6* than in *rev-6* (Fig. 2G). The SAM-defective plants included those where: (i) the SAM was replaced by a needle-like structure (Fig. 2B) or completely terminated (Fig. 2C, D) after producing two rosette leaves; (ii) the SAM was terminated and the whole seedling was arrested after cotyledons opened (Fig. 2E); and (iii) the SAM was replaced by a single cotyledon (Fig. 2F).

After bolting, primary inflorescences of wild-type (data not shown) and most *rev-6* single mutant (Fig. 2H) plants usually produced several branches, each associated with a cauline leaf at the proximal end. However, up to 96% of the *ae4-1 rev-6* plants form bare axils, albeit with the presence of cauline leaves (Fig. 2I), which is much higher than the number found in the *rev-6* single mutant plants (Fig. 2L). Scanning electron microscopy (SEM) (Fig. 2J) and longitudinal section (Fig. 2K) through the stem–branch junction region revealed that the AM structure was missing completely, as previously reported in the *rev* single mutants (Talbert *et al.*, 1995; Otsuga *et al.*, 2001). These results, together with the previously reported analyses in *hlr/rpt2a* (Ueda *et al.*, 2004) and *ae3/rpn8a rev* double mutants (Huang *et al.*, 2006), suggest that the 26S proteasome has a function in regulating meristem maintenance.

ae4 rev showed severe inflorescence and flower developmental defects

The reproductive development of the *ae4-1 rev-6* double mutant was also analysed. Compared with wild-type plants (Fig. 3A), the *ae4-1* (Fig. 3B) and *rev-6* (Fig. 3C) single mutants both produced flowers normally, although the phyllotaxy in *ae4-1* was not the normal spiral form (Fig. 3B). Inflorescences of a proportion of *ae4-1 rev-6* plants were arrested after producing a few normal-looking flowers (Fig. 3D) and varying numbers of sterile flowers, which were morphologically aberrant. The normal-looking flowers formed fertile siliques (Fig. 3D), whereas the other flowers either showed altered shapes of organs or developed to filamentous structures (Fig. 3E, arrows). In some extreme cases, one *ae4-1 rev-6* plant produced only a sole terminal flower at the apex of the inflorescence, together with many filamentous organs (Fig. 3F). During late reproductive development, while some double mutant

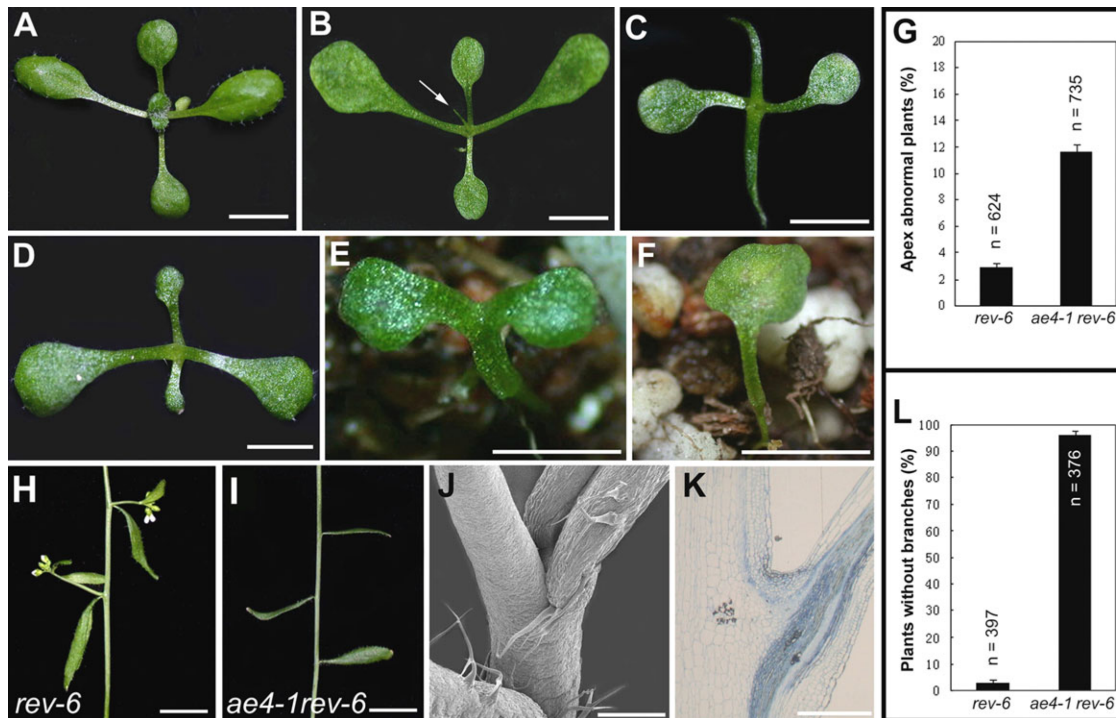

Fig. 2. The double mutant *ae4 rev* displayed enhanced SAM defects of *rev*. (A–F) Phenotypes of *ae4-1 rev-6* seedlings. Most *ae4-1 rev-6* plants had a normal vegetative SAM (A), but a proportion of them showed a variety of vegetative SAM defects. The abnormalities of the vegetative SAM include: it was replaced by a needle-like structure (B); was terminated after the first pair of rosette leaves (C and D) or when the cotyledons (E) were expanded; or was replaced by a single cotyledon (F). Although some *rev-6* mutant plants also showed SAM defects, the number of such plants was much greater in *ae4-1 rev-6* than in *rev-6* (G). Compared with *rev-6* plants (H), most *ae4-1 rev-6* plants did not produce axillary branches (I), and the number of such plants was much greater than that of *rev-6* (L). SEM (J) and longitudinal section (K) showed that the AM was missing in the *ae4-1 rev-6* plants. Bars=0.5 cm in A–I, and 0.1 mm in J and K.

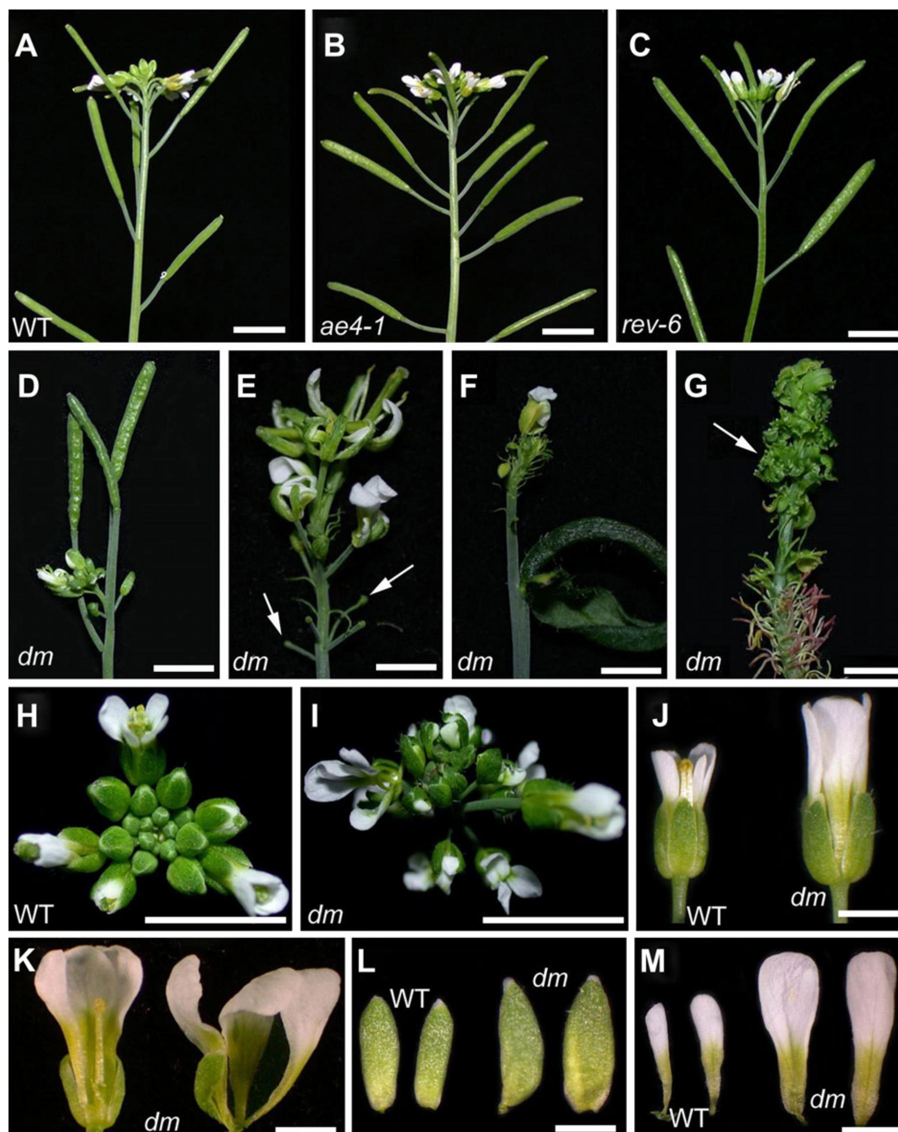

Fig. 3. Reproductive developmental defects in the *ae4 rev* double mutant. (A–G) Inflorescence phenotypes of the wild type (A), *ae4-1* (B), *rev-6* (C), and *ae4-1 rev-6* (D–G). Note that the *ae4-1 rev-6* plants had the earlier terminated apex (D–G), distorted floral shapes (E), and filamentous structures (F). In very late reproductive development, *ae4-1 rev-6* plants only produce carpel-like organs and filamentous structures (G). (H and I) A top view of wild-type (H) and *ae4-1 rev-6* (J) inflorescences, showing that *ae4-1 rev-6* displayed an unusual floral phyllotaxy. Several earlier appearing flowers of *ae4-1 rev-6* looked normal, but these flowers were larger compared with wild-type flowers (J), with large sepals (L) and petals (M). (K) The *ae4-1 rev-6* flowers usually produced a reduced number of floral organs. dm, *ae4-1 rev-6* double mutant. Bars=0.5 cm in A–I, and 0.2 cm in J–M.

plants were arrested after producing one or several flowers and varying numbers of filamentous organs, other plants continued to generate carpelloid structures (Fig. 3G; Supplementary Fig. S2 at *JXB* online). Compared with the wild-type plants (Fig. 3H), phyllotaxy in most *ae4-1 rev-6* plants was disrupted (Fig. 3I). The normal-looking flowers of *ae4-1 rev-6* were usually larger (Fig. 3J), with the outer two whorl organs, sepals (Fig. 3L), and petals (Fig. 3M) all enlarged. Compared with the wild type, the aberrant *ae4-1 rev-6* flowers often exhibited reduced floral organ numbers (Fig. 3K and Table 1). In some cases, the inner two whorls of *ae4-1 rev-6* flowers contained only one stamen or were completely lacking organs (Fig. 3K).

Table 1. The *ae4-1 rev-6* flowers produced reduced numbers of floral organs

Genotype	No. of floral organs per flower[a]			
	Sepal	Petal	Stamen	Carpel
ae4-1 (*n*=30)	4.0±0	4.0±0	5.7±0.5	2.0±0
rev-6 (*n*=36)	4.0±0	4.0±0	5.8±0.4	2.0±0
ae4-1 rev-6 (*n*=31)	2.5±0.6	2.6±1.0	0.1±0.3	NA[b]

[a] The 6th–10th flowers were counted for *ae4-1*, *rev-6*, and *ae4-1 rev-6* plants.
[b] Not available.

To understand better the roles of *AE4* during flower development, *ae4-1 rev-6* inflorescences were analysed using plants on days 4 and 12 after flowering, by examining longitudinal sections. Compared with those of wild-type plants (Fig. 4A), the day 4 inflorescences of *ae4-1 rev-6* were able to produce some floral primordia that subsequently generated different types of floral organs (Fig. 4B). However, the day 12 *ae4-1 rev-6* inflorescence gave rise to floral primordia that were either arrested after emergence or developed to filamentous structures (Fig. 4C). The *ae4-1 rev-6* inflorescences and floral primordia were then investigated using SEM. The young flowers of the wild type (Fig. 4D), *ae4-1* (Fig. 4E), and *rev-6* (Fig. 4F) had no obvious differences in the shape and size of their IM at day 4 and day 12 (data not shown). In contrast, although the shape and size of the IM appeared normal in day 4 inflorescences of most *ae4-1 rev-6* plants, some flowers were observed to lack one or two sepals (Fig. 4G, arrow). In a small proportion of double mutant plants, the day 4 inflorescence generated very few flowers but many primordia, which might have formed the filamentous structures (Fig. 4H). On day 12 after flowering, the *ae4-1 rev-6* inflorescence only produced filamentous structures or primordia that were arrested after emergence (Fig. 4I). In some flowers of the double mutant with the reduced number of sepals, the remaining sepals did not wrap the inner organs correctly (Fig. 4J). Some floral primordia appeared to have four sepal primordia, but the FM did not develop accordingly, resulting in some flowers with a structure similar to that in the *wus* mutant flowers (Fig. 4K, arrow) (Laux *et al.*, 1996; Mayer *et al.*, 1998).

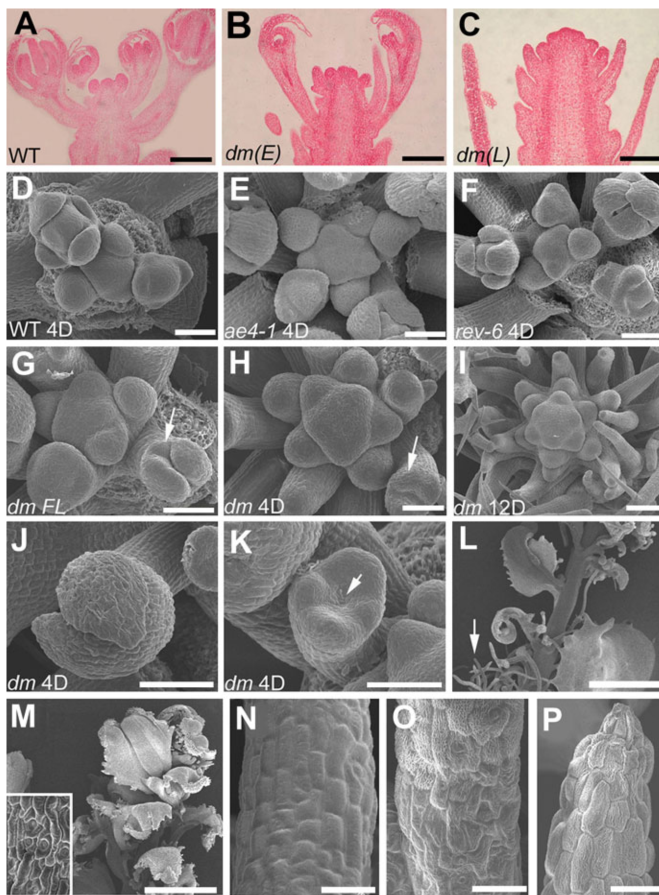

Fig. 4. Sectioning and SEM analyses of inflorescence and flower phenotypes of the *ae4-1 rev-6* double mutant. (A–C) Longitudinal sections of inflorescences from wild-type (A) and *ae4-1 rev-6* plants at an early stage (4 d after flowering) (B), and a relatively late stage (12 d after flowering) (C). (D–F) The IM structures of L*er* (D), *ae4-1* (E), and *rev-6* (F). (G–I) The IMs of *ae4-1 rev-6* at the time of flowering (G), or on day 4 (H) and day 12 (I) after flowering. *FL*, the inflorescence was fixed on the day when the first flower was open. (J) An aberrant *ae4-1 rev-6* flower with reduced numbers of sepals, which incorrectly surrounded the inner floral organs. (K) An *ae4-1 rev-6* floral primordium, from which four sepal primordia had emerged but the floral meristem was terminated. (L, M) The inflorescence of *ae4-1 rev-6* generated carpelloid organs after producing filamentous structures during late reproductive development, and the carpelloid organs contained stomata cells in the abaxial epidermis (M, inset). (N–P) The epidermis on the bottom part of the *ae4-1 rev-6* filamentous structures contained mixed long/narrow and rectangular cells (N), while the epidermal cells in the distal part mimicked the abaxial sepal and petal surface cells, which are cobblestone-shaped with wavy lines on the cell surfaces (O, P). dm, *ae4-1 rev-6* double mutant. Bars=50 μm in A–K, 1 mm in L and M, and 20 μm in N–P.

While about a half of the *ae4-1 rev-6* plants grown under the present conditions showed arrested inflorescence after producing one to several flowers and different numbers of filamentous structures, inflorescences of other double mutant plants continued to generate carpelloid organs (Fig. 4L, M). In addition, numerous stomata cells were found in the abaxial epidermis of the carpelloid organs (Fig. 4M, inset). In wild-type plants, the filament of stamens is covered with long and narrow epidermal cells (data not shown). In *ae4-1 rev-6*, however, the epidermis on the bottom part of the filamentous structure contained mixed long/narrow and rectangular cells (Fig. 4N). In the distal part of this structure, epidermal cells of the *ae4-1 rev-6* filamentous structures mimicked those on the abaxial sepal and petal surface cells, which are cobblestone-shaped with wavy lines on the cell surfaces (Fig. 4O, P). Taken together, these results indicate that during late reproductive development, the *ae4-1 rev-6* IM and FM lose their functions gradually during flower and floral organ development.

AE4 is required for normal WUS and CLV expression in the IM and FM

As some *ae4-1 rev-6* flowers resemble those observed in the *wus* mutant (Figs 3K, 4K) (Laux *et al.*, 1996; Mayer *et al.*, 1998), and *WUS* and *CLV* form a feedback loop to maintain meristem activities, expression patterns of *WUS* and *CLV3* were analysed in the *ae4-1 rev-6* IM and FM. *In situ* hybridization showed that *WUS* transcripts were detected in the IM and floral primordia in the wild type (Fig. 5A), consistent with previous results (Mayer *et al.*, 1998). However, although the *WUS* expression pattern appeared normal in the day 4 *ae4-1 rev-6* IMs and in some

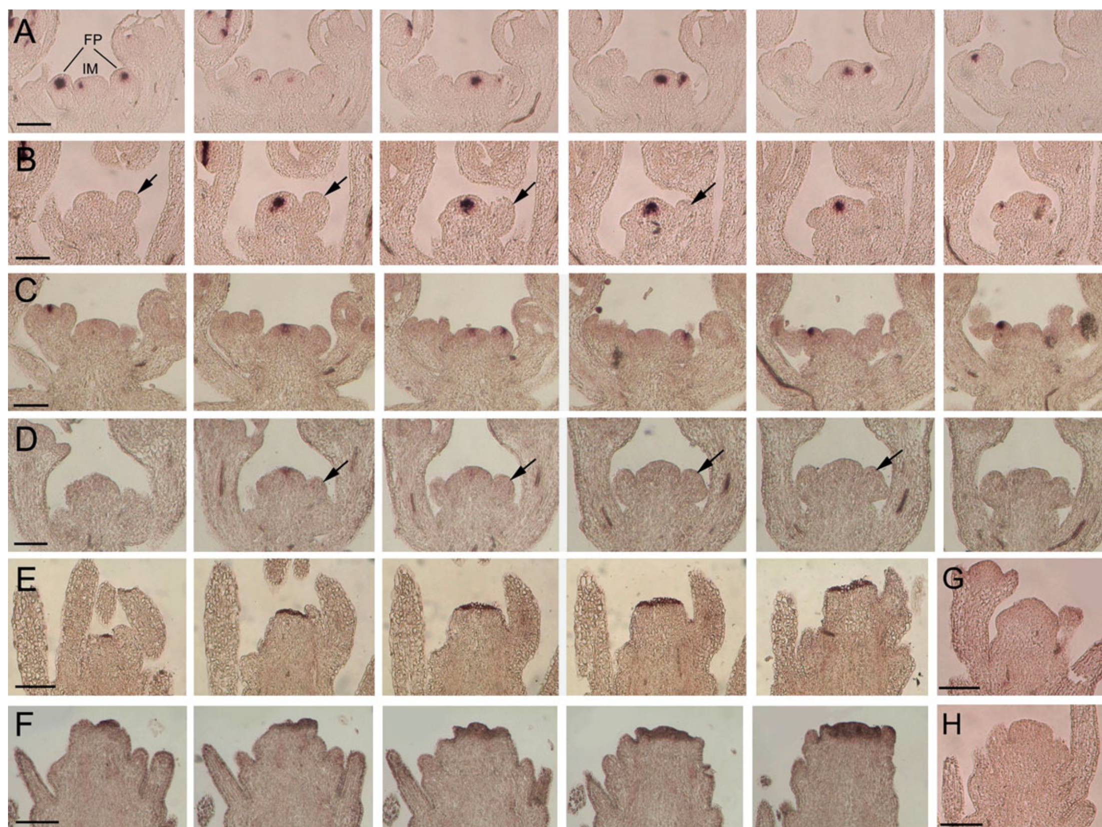

Fig. 5. *In situ* hybridization to detect *WUS* and *CLV3* transcripts during *ae4-1 rev-6* flower development. (A–F) Serial sections of wild type (A and C) and *ae4-1 rev-6* (B, D, and F) inflorescences, during early (A–D) and late (E–H) stages of reproductive development. (A and B) Wild-type (A) and *ae4-1 rev-6* (B) inflorescences were detected with the *WUS* antisense probe. (C and D) Wild-type (C) and *ae4-1 rev-6* (D) inflorescences were detected with the *CLV3* antisense probe. (E and F) The *ae4-1 rev-6* inflorescences were detected with *WUS* and *CLV3* antisense probes, respectively. (G and H) The *ae4-1 rev-6* inflorescences were detected with *WUS* and *CLV3* sense probes, respectively. IM, inflorescence meristem; FP, floral primordia. Arrows in B and D indicate that some *ae4-1 rev-6* floral primordia did not express *WUS* and *CLV3*, respectively. Bars=20 μm in A–H.

floral primordia (Supplementary Fig. S3 at *JXB* online), the *WUS* signals were absent in many other same-stage floral primordia (Fig. 5B). In addition, the *WUS* signal-containing region in the day 4 *ae4-1 rev-6* IM seemed broader: not only was the *WUS* signal present in four successive sections in *ae4-1 rev-6* (Fig. 5B) versus three in the wild type (Fig. 5A), but the signal range was also closer to the IM tip in *ae4-1 rev-6* than in the wild type.

Similarly to previous results (Fletcher *et al.*, 1999), *CLV3* was expressed in the central zone above *WUS*-expressing cells in the IM and in the FM of flowers of stages 1–6 (Fig. 5C). The *CLV3* expression pattern in most day 4 *ae4-1 rev-6* IMs (Fig. 5D) and some FMs (Supplementary Fig. S3 at *JXB* online) appeared normal. However, the *CLV3* transcripts were not detected in some other FMs in *ae4-1 rev-6* floral primordia (Fig. 5D). Interestingly, in some day 12 *ae4-1 rev-6* IMs, both *WUS* (Fig. 5E) and *CLV3* (Fig. 5F) transcripts strongly accumulated in the top-layer cells, whereas the respective sense probes did not yield hybridization signals (Fig. 5G, H). These results suggest that altered *WUS* and *CLV3* expression correlated, at least partially, with the inflorescence and flower phenotypes of *ae4-1 rev-6* during reproductive development.

The 26S proteasome might act as a whole in maintaining the IM and FM

To provide additional evidence that the 26S proteasome pathway regulates the IM and FM functions, double mutants were constructed using other 26S proteasome subunit mutants as well as another *rev* allele, *rev-9*. Analysis of these mutants showed that, similarly to *ae4-1 rev-6*, the *ae4-2 rev-6* plants also lacked axillary branches (Fig. 6A), with an arrested inflorescence (Fig. 6B). In addition, some flowers showed incorrectly organized sepals and did not contain the inner floral organs (Fig. 6C). Occasionally, *ae4-2 rev-6* plants also produced an inflorescence that only contained filamentous structures (Fig. 6D). The double mutant *ae4-1 rev-9* was similar to *ae4-1 rev-6* (Fig. 6E). Phenotypes of the *rpn1a-2* and *rpn9a-1* single mutants were indistinguishable from those of wild-type plants at both the vegetative and reproductive developmental stages. However, phenotypes of the *rpn1a-2 rev-6* and *rpn9a-1 rev-6* double mutants generally resembled those of *ae4-1 rev-6*. For example, these double mutant plants lacked the axillary branch (Fig. 6F, H), and inflorescences of some double mutant plants were terminated after producing a few flowers that set seeds

Fig. 6. The phenotypes of *ae4-2 rev-6*, *ae4-1 rev-9*, *rpn1a-2 rev-6*, and *rpn9a-1 rev-6* are generally similar to those of *ae4-1 rev-6*. (A) An *ae4-2 rev-6* inflorescence. An inset image in the right shows a cauline leaf with a bare axil. (B) Close-up of the inflorescence apex in (A), showing abnormal floral phyllotaxy. (C) Close-up of a flower in (B), showing incorrectly arranged sepals and petals. (D) Occasionally, some *ae4-2 rev-6* plants had an inflorescence that only produced filamentous structures. (E–H) Inflorescences of *ae4-1 rev-9* (E), *rpn1a-2 rev-6* (F), and *rpn9a-1 rev-6* (G, H) are similar overall to *ae4-1 rev-6*, although the phenotypic severity was variable. An inset image in the right in (F) shows a cauline leaf with a bare axil, and an arrowhead indicates the weakly affected inflorescence. Bars=1 cm in A and D–H, 0.5 cm in B, and 0.2 cm in C.

(Fig. 6G). In addition, the *rpn9a-1 rev-6* inflorescences also produced filamentous structures (Fig. 6G, H). These data suggest that the 26S proteasome genetic pathway is involved in regulating IM and FM functions.

Discussion

The 26S proteasome is an essential protein complex for living eukaryotic organisms. In *Arabidopsis*, most 26S proteasome subunits are encoded by two copies of isoforms (Smalle and Vierstra, 2004). For many 26S proteasome subunit genes, mutation in one of them usually results in relatively weak phenotypic changes, whereas plants with double mutations in both isoforms are often non-viable (Huang *et al.*, 2006; Book *et al.*, 2009; Gallois *et al.*, 2009). One important discovery regarding the 26S proteasome function in development was made by identification and characterization of the *Arabidopsis hlr* mutant: the *HLR* function was proposed to regulate both the root apical meristem (RAM) and SAM (Ueda *et al.*, 2004). The phenotypes of the *ae3-1/rpn8a rev-9* double mutant were previously reported, showing that the plants were almost all arrested at the seedling stages. This result indicated that the 26S proteasome and the *REV* gene are required for maintenance of the vegetative SAM, consistent with the proposal raised by analysis of the *hlr* mutant. However, because of the strong phenotypes of *ae3-1/rpn8a rev-9*, whether they are also required for the maintenance of the IM and FM and how they function in reproductive development are largely unknown. In this work, it was shown that the double mutant *ae4-1 rev-6* is able to pass through vegetative development until the late reproductive developmental stages, and thus the 26S proteasome and *REV* functions in regulating the IM and FM could be investigated.

The results revealed that, in addition to the RAM and vegetative SAM (Ueda *et al.*, 2004; Huang *et al.*, 2006), the 26S proteasome is also required for maintenance of the AM, IM, and FM. At vegetative developmental stages, the numbers of plants with an abnormal vegetative SAM and that were AM free in *ae4-1 rev-6* were much greater than those in *rev-6*. At reproductive developmental stages, about a half of the *ae4-1 rev-6* plants were arrested after producing some flowers and filamentous organs, due to the aberrant IMs, whereas no such plants were observed in the *rev-6* single mutant. Finally, a large number of *ae4-1 rev-6* flowers showed reduced numbers of floral organs, indicating abnormal FMs. All these results indicate that the 26S proteasome function is generally required for the maintenance of various kinds of meristems. As the proteolytic function of the 26S proteasome is critical for cell cycle progression by degradation of important cell cycle regulators (Criqui and Genschik, 2002; Reed, 2003; Smalle and Vierstra, 2004; Inze and De Veylder, 2006), the function of the 26S proteasome in normal cell cycle control might be important in the maintenance of different meristematic tissues.

The present results also reveal a new function for the 26S proteasome and the *REV* pathways in specifying IM and FM identities. In reproductive development, the IM generates floral primordia that subsequently show FM functions, leading to the formation of floral organs. At the

molecular level, the *WUS*-*CLV* network is active in the IM-generated floral primordia, but not in the FM-generated floral organ primordia (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). In the *ae4-1 rev-6* double mutant, although during the early reproductive developmental stages the IM could produce a few primordia that formed normal-looking flowers, at later stages the original *ae4-1 rev-6* IM was transformed into a mixed IM/FM structure. The inflorescence stem continued to elongate, manifesting a wild-type stem, whereas the mixed IM/FM structure only produced flower organ-like structures, but not flowers. These organs include: (i) filamentous structures that were covered with mixed filament cells of stamens and abaxial cells of sepals and petals; and (ii) carpelloid organs, which were associated with stigmatic tissues. These phenotypes appeared at the late reproductive developmental stages, suggesting that at these stages plants are more sensitive to differences in the 26S proteasome and *REV* function.

The *ae4 rev* double mutant showed some plant phenotypes similar to those seen in the *wus* mutant (Laux *et al.*, 1996; Mayer *et al.*, 1998). For example, certain double mutant plants showed embryonic SAM defects, arresting at the cotyledon stage or having a single cotyledon that replaced the SAM. In addition, some flowers of the *ae4 rev* double mutant lacked the inner whorl floral organs. During flower development, *WUS* and *CLV* genes are expressed in the floral primordium to maintain the FM, but their transcripts were not detected in the late-stage *ae4-1 rev-6* floral primordia, further supporting the premise that these primordia lack the ability to become flowers. In addition, floral organ development is independent of the *WUS*-*CLV* pathways. The late-stage primordia produced by the *ae4-1 rev-6* mixed IM/FM structure failed to express *WUS* and *CLV*, and thus may be one of the reasons why the primordia can only develop into certain floral organ-like structures. The fact that both *CLV* and *WUS* activities were severely reduced in floral primordia of the *ae4-1 rev-6* plants is consistent with the previous proposal that *REV* acts upstream of the *CLV* loci (Otsuga *et al.*, 2001). The present data also suggest that in addition to *REV*, the 26S proteasome may also act upstream of the *WUS*-*CLV* pathway and plays a role in promoting this pathway.

In severe cases, some flowers of the *ae4-1 rev-6* double mutant did not initiate any floral organ, especially for the late appearing floral primordia. Such abnormal phenotypes cannot be completely explained by the down-regulation of the *WUS* gene, because flowers of the null *wus-1* allele could form some sepals and petals, and sometimes even a stamen, in the centre of a defective flower (Laux *et al.*, 1996; Mayer *et al.*, 1998). One possibility is that *REV* and the 26S proteasome together regulate other pathway(s) required for lateral organ initiation. It was generally thought that a local maximum of plant hormone auxin, generated by the PINFORMED1 (PIN1) auxin efflux carrier, is essential in lateral organ initiation (Reinhardt *et al.*, 2000, 2003; Benkova *et al.*, 2003). The 26S proteasome is known to play critical roles in auxin signalling by degradation of AUX/IAA transcriptional repressors and thus affects auxin-modulated transcriptional regulation (Parry and Estelle, 2006). Recent results also showed that the expression pattern of *PIN4* was notably altered in the *rpn5a* mutant, which corresponds to another subunit of the 26S proteasome (Book *et al.*, 200). On the other hand, *REV* is known to have functions in regulating multiple developmental processes, and some of these functions are tightly linked to auxin signalling and transport (Zhong and Ye, 2001; Mattsson *et al.*, 2003; Izhaki and Bowman, 2007; Ilegems *et al.*, 2010). Thus, failure to initiate floral organ primordia in the *ae4-1 rev-6* double mutant plants might also be linked to auxin transport and signalling.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Identification of *ae4-2* (SALK_133787).

Figure S2. Additional images of *ae4-1 rev-6* plants during late stage of reproductive development, showing numerous filamentous organs and carpelloid structures. Bars = 0.5 cm.

Figure S3. *In situ* hybridization detection of *WUS* (A) and *CLV3* (B) transcripts in the normal-looking flowers in the day 4 infloresences of *ae4-1 rev-6* plants. Bars = 20 μm in A and B.

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