RESEARCH PAPER

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

Zhenzhen Zhang, Hua Wang, Dexian Luo, Minhuan Zeng, Hai Huang and Xiaofeng Cui*

National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China

* To whom correspondence should be addressed. E-mail: xiaofeng@sippe.ac.cn

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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 CLVI, 2, and 3, which encode a leucine-rich repeat (LRR)-receptor kinase, an LRR receptor-like protein, and a small secreted peptide,
respectively (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999). The chl1, 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; Trotouchaud 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. 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-irn2a, 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 from 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, Ler) 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 Ler three times before detailed phenotypic analyses. The rev-6 and rev-9 alleles, both in the Ler 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.

Complementation analysis of ae4-2 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 Ler; 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’-TAACAAAGCCTATCCCCAGC-3’ and 5’-GCTTTATCCCGAGGC-3’ for WUS; and 5’-TCCAGATCTCCTCAAG-3’ and 5’-CTTACATTCCTGCAA-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 dissection 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 LEAVES12 (ASI) and AS2 regulatory network, a single as2-101 enhancer mutant, designated as ae4-1 (asymmetric leaves12 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...
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 F1 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
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.
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 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.

| Genotype           | 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     |

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

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).

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
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 \( \text{ae4-1 rev-6} \) IM seemed broader: not only was the WUS signal present in four successive sections in \( \text{ae4-1 rev-6} \) (Fig. 5B) versus three in the wild type (Fig. 5A), but the signal range was also closer to the IM tip in \( \text{ae4-1 rev-6} \) 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 \( \text{ae4-1 rev-6} \) 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 \( \text{ae4-1 rev-6} \) floral primordia (Fig. 5D). Interestingly, in some day 12 \( \text{ae4-1 rev-6} \) 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 \( \text{ae4-1 rev-6} \) 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 \( \text{ae4-1 rev-6} \), the \( \text{ae4-2 rev-6} \) 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, \( \text{ae4-2 rev-6} \) plants also produced an inflorescence that only contained filamentous structures (Fig. 6D). The double mutant \( \text{ae4-1 rev-9} \) was similar to \( \text{ae4-1 rev-6} \) (Fig. 6E). Phenotypes of the \( \text{rpn1a-2} \) and \( \text{rpn9a-1} \) single mutants were indistinguishable from those of wild-type plants at both the vegetative and reproductive developmental stages. However, phenotypes of the \( \text{rpn1a-2 rev-6} \) and \( \text{rpn9a-1 rev-6} \) double mutants generally resembled those of \( \text{ae4-1 rev-6} \). 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

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**Fig. 5.** In situ hybridization to detect WUS and CLV3 transcripts during \( \text{ae4-1 rev-6} \) flower development. (A–F) Serial sections of wild type (A and C) and \( \text{ae4-1 rev-6} \) (B, D, and F) inflorescences, during early (A–D) and late (E–H) stages of reproductive development. (A and B) Wild-type (A) and \( \text{ae4-1 rev-6} \) (B) inflorescences were detected with the WUS antisense probe. (C and D) Wild-type (C) and \( \text{ae4-1 rev-6} \) (D) inflorescences were detected with the CLV3 antisense probe. (E and F) The \( \text{ae4-1 rev-6} \) inflorescences were detected with WUS and CLV3 antisense probes, respectively. (G and H) The \( \text{ae4-1 rev-6} \) inflorescences were detected with WUS and CLV3 sense probes, respectively. IM, inflorescence meristem; FP, floral primordia. Arrows in B and D indicate that some \( \text{ae4-1 rev-6} \) floral primordia did not express WUS and CLV3, respectively. Bars=20 μm in A–H.
In addition, the \textit{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 \textit{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 \textit{et al.}, 2006; Book \textit{et al.}, 2009; Gallois \textit{et al.}, 2009). One important discovery regarding the 26S proteasome function in development was made by identification and characterization of the \textit{Arabidopsis hlr} mutant: the \textit{HLR} function was proposed to regulate both the root apical meristem (RAM) and SAM (Ueda \textit{et al.}, 2004). The phenotypes of the \textit{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 \textit{REV} gene are required for maintenance of the vegetative SAM, consistent with the proposal raised by analysis of the \textit{hlr} mutant. However, because of the strong phenotypes of \textit{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 \textit{ae4-1 rev-6} is able to pass through vegetative development until the late reproductive developmental stages, and thus the 26S proteasome and \textit{REV} functions in regulating the IM and FM could be investigated.

The results revealed that, in addition to the RAM and vegetative SAM (Ueda \textit{et al.}, 2004; Huang \textit{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 \textit{ae4-1 rev-6} were much greater than those in \textit{rev-6}. At reproductive developmental stages, about a half of the \textit{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 \textit{rev-6} single mutant. Finally, a large number of \textit{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 \textit{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-l 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 rpm5a mutant, which corresponds to another subunit of the 26S proteasome (Book et al., 2000). 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 inflorescences of ae4-1 rev-6 plants. Bars = 20 μm in A and B.

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References

Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591–602.

Book AJ, Smalle J, Lee KH, et al. 2009. The RPN5 subunit of the 26S proteasome is essential for gametogenesis, sporophyte development, and complex assembly in Arabidopsis. The Plant Cell 21, 460–478.

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

Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R. 2000. Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science 289, 617–619.

Chen CB, Wang SP, Huang H. 2000. LEUNIG has multiple functions in gynoecium development in Arabidopsis. Genesis 26, 42–54.
divisions in the leaves and stems of Arabidopsis thaliana. Development 121, 2723–2735.

Trotochaud AE, Hao T, Wu G, Yang Z, Clark SE. 1999. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. The Plant Cell 11, 393–406.

Ueda M, Matsui K, Ishiguro S, Sano R, Wada T, Paponov I, Palme K, Okada K. 2004. The HALTED ROOT gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of Arabidopsis meristems. Development 131, 2101–2111.

Xu L, Xu Y, Dong A, Sun Y, Pi L, Huang H. 2003. Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMMETRIC LEAVES1 and 2 and ERECTA functions in specifying leaf adaxial identity. Development 130, 4097–4107.

Xu Y, Sun Y, Liang WQ, Huang H. 2002. The Arabidopsis AS2 gene encoding a predicted leucine-zipper protein is required for the leaf polarity formation. Acta Botanica Sinica 44, 1194–1202.

Zhong R, Ye ZH. 2001. Alteration of auxin polar transport in the Arabidopsis ifl1 mutants. Plant Physiology 126, 549–563.