VAJ/GFA1/CLO is Involved in the Directional Control of Floral Organ Growth

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Flowers assume variant forms of reproductive structures, a phenomenon which may be partially due to the diversity among species in the shape and size of floral organs. However, the organ size and shape of flowers usually remain constant within a species when grown under the same environmental conditions. The molecular and genetic mechanisms that control organ size and shape are largely unknown. We isolated an *Arabidopsis* mutant, *vajra-1* (*vaj-1*), exhibiting defects in the regulation of floral organ size and shape. In *vaj-1*, alterations in the size and shape of floral organs were caused by changes in both cell size and cell number. The *vaj-1* mutation also affected the number of floral organs. In *vaj-1*, a mutation was found in *GAMETOPHYTIC FACTOR 1 (GFA1)/CLOTHO (CLO)*, recently shown to be required for female gametophyte development. The *VAJ/GFA1/CLO* gene encodes a translational elongation factor-2 (EF-2) family protein, of which the human U5-116kD and yeast Snu114p counterparts are U5 small nuclear ribonucleoprotein (snRNP)-specific proteins. A transient expression assay using *Arabidopsis* protoplasts revealed that VAJ protein co-localized with SC35, a serine/arginine-rich (SR) protein involved in pre-mRNA splicing. Our results showed that *VAJ/GFA1/CLO* has a novel role in the directional control of floral organ growth in *Arabidopsis*, possibly acting through pre-mRNA splicing.

Keywords: *Arabidopsis thaliana* • Directional organ growth • Floral organ development • Pre-mRNA splicing.

Abbreviations: BAC, bacterial artificial chromosome; Ds-RED, *Discosoma* sp. red fluorescent protein; EF-2, translational elongation factor-2; GFP, green fluorescent protein; GUS, β-glucuronidase; mRFP, monomeric red fluorescent protein; NMD, nonsense-mediated decay; ORF, open reading frame; PTC, premature translation termination codon; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; snRNP, small nuclear ribonucleoprotein; SR, serine/arginine-rich; UTR, untranslated region.

Introduction

Arabidopsis flowers are composed of four types of floral organs: four sepals, four petals, six stamens and two-fused carpels forming a gynoecium. In a single flower, these four types of organs are arranged in a concentric manner. First, sepal primoridia appear on the flanks of a floral meristem and grow around it, finally enclosing the flower meristem. Other floral organ primordia arise and develop inside floral buds (Smyth et al. 1990). Floral organ primordia assume different identities based on the combined function of several floral organ identity genes (Zik and Irish 2003, Krizek and Fletcher 2005).

Plant cells are surrounded by a rigid cell wall, which prevents movement of the plant cell during organ development. In this context, control of cell division rate, and the pattern and regulation of polarized cell growth play important roles in plant organogenesis (Meyerowitz 1997). To date, several cases have been reported in which mutations affecting the control of cell proliferation or cell growth evoked changes in organ size and shape.

AINTEGUMENTA (ANT), an AP2-domain family transcription factor, was shown to control cell proliferation positively by maintaining the meristematic competence of the cell (Elliott et al. 1996, Klucher et al. 1996, Krizek 1999,

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Mizukami and Fischer 2000). In *ant* mutants, the number and size of floral organs were reduced due to fewer cells, while floral and vegetative organs were larger than those of the wild type in *ANT*-overexpressing plants, 35S::ANT, mainly due to an increase in cell number. *BIG BROTHER* (*BB*), which encodes an E3 ubiquitin ligase, was shown to restrict growth in the stem and lateral organs by limiting the duration of cell proliferation (Disch et al. 2006).

Once primordia of lateral organs, floral organs and leaves are formed, their growth progresses along three axes: the proximal–distal (longitudinal), central–lateral (transverse) and adaxial–abaxial axes. Directional controls of cell proliferation and cell elongation along the proximal–distal axis and the central–lateral axis were identified as contributing to the determination of organ shape during organ development. *ROTUNDIFOLIA4* (*ROT4*) encodes a membrane-bound small peptide, and *rot4-1D* mutants ectopically expressing higher levels of *ROT4* had short leaves, an alteration caused by reduced cell proliferation specifically in the longitudinal direction of leaves (Narita et al. 2004). Mutations in *ANGUSTIFOLIA* (*AN*), which encodes a protein with similarity to CtBP/BARS family proteins, render leaves narrower than those of the wild type due to diminished cell elongation in the transverse direction (Tsuge et al. 1996, Folkers et al. 2002, Kim et al. 2002). On the other hand, *rotundifolia3* (*rot3*) mutants show defects in polar cell elongation in the longitudinal direction (Tsukaya et al. 1995, Tsuge et al. 1996). *ROT3* encodes a cytochrome P450 (CYP90C1) which is involved in brassinosteroid biosynthesis (Kim et al. 1998, Kim et al. 1999, Kim et al. 2005). Two homologous genes, *LONGIFORIA1* (*LNG1*) and *LONGIFOLIA2* (*LNG2*), encoding proteins with nuclear localization signals and with unknown molecular function, also promote polar cell elongation in the longitudinal direction, independently of *ROT3* (Y. K. Lee et al. 2006). These genes were shown to regulate polarized cell proliferation and cell elongation only in a single direction. Maintenance of an appropriate balance of directional organ growth between longitudinal and transverse axes is required to produce a consistent organ size and shape. However, it is not clear whether these two separate axis-dependent controls interact to ensure proper lateral organ size and shape, or whether there are genes which act to control both longitudinal and transverse directional organ growth by regulating the functions of known genes required for directional organ growth.

It was reported that defects in pre-mRNA splicing affected the control of organ size in lateral organ development. The *BIG PETAL* (*BPE*) locus was involved in petal size control (Szecsi et al. 2006). Transcripts from *BPE* were alternatively spliced and generated two types of transcripts, which encode different types of basic helix–loop–helix (bHLH) transcription factors. Longer transcripts, *BPEp*, were preferentially expressed in petals, while the expression of shorter transcripts,

BPEub, was ubiquitously detected in several organs including flowers. Mutation and RNA interference (RNAi) experiments which reduced *BPEp* expression were shown to increase petal size because of enlarged petal cells (Szecsi et al. 2006). This indicates that proper control of gene expression by alternative splicing is required for determination of petal size. In addition to control via alternative splicing, precise control of mRNA metabolism by constitutive pre-mRNA splicing machinery has also been shown to be required for the control of lateral organ size and shape. SR45, a plant-specific splicing factor, was isolated as the interactant with U1-70K (Golovkin and Reddy 1999). In *sr45-1* mutants, the proportion of length/width of leaves and petals was altered, and the number of petals and stamens was affected (Ali et al. 2007). The *SWELLMAP1* (*SMP1*) gene encodes a CCHC zinc finger protein similar to yeast step II splicing factor, Slu7 (Clay and Nelson 2005). The *SMP1* gene was shown to be highly expressed in the region of cell proliferation. Accordingly, in the *smp^{epi}* mutant, whose expression of *SMP1* and *SMP2* was decreased, the duration of cell proliferation of leaves was shortened, and as a result the leaf size of the *smp^{epi}* mutants was smaller than that of the wild type.

To gain more insight about the directional control of floral organ growth, we isolated and analyzed an *Arabidopsis* mutant, *vajra* (*vaj*). In contrast to other known mutations affecting directional organ growth, the *vaj-1* mutation produce antagonistic effects on floral organ development in the longitudinal and transverse directions. In this mutant, floral organ width was reduced, though floral organ length was increased, owing to the defects in the regulation of cell proliferation and polar cell growth. In addition, an alteration in the number of floral organs and the partial fusion of floral organs were observed in *vaj-1*. The *VAJ/GFA1/CLO* gene encodes a translational elongation factor-2 (EF-2) family protein, which shows similarity to human U5-116kD and yeast Snu114p, factors known to be components of the pre-mRNA splicing machinery (Fabrizio et al. 1997, Bartels et al. 2002, Coury et al. 2007, Moll et al. 2008). We found that VAJ/GFA1/CLO protein co-localized with a serine/arginine-rich (SR) protein SC35 in the nuclei. These results demonstrated a novel function for *VAJ/GFA1/CLO* in controlling floral organ size, probably by way of pre-mRNA metabolism.

Results

The vaj-1 mutant shows defects in the control of the size of floral organs

We isolated a recessive mutant, *vaj-1*, showing defects in the control of floral organ size. We named this mutant *vajra* (*vaj*), because the shape of a flower bud when ready to open resembles VAJRA, an instrument used in esoteric Buddhism. Sepals of *vaj-1* failed to enclose flower buds, and therefore

the insides of flowers are visible (**Fig. 1A**, right), implying that *vaj-1* sepals are narrower than those of the wild type, Landsberg erecta (L*er*). Compared with that of the wild type, the *vaj-1* sepal width was decreased to about 65% (wild type, 0.56 ± 0.07 mm; *vaj-1*, 0.36 ± 0.09 mm; mean ± SD evaluated by Student's paired *t*-test at $P < 0.001$, **Fig. 1B**), while *vaj-1* sepal length was increased up to about 1.4-fold (wild type, 1.50 ± 0.24 mm; *vaj-1*, 2.06 ± 0.41 mm; $P < 0.001$, **Fig. 1B**). These data showed that *vaj-1* sepals are narrower and longer

than those of the wild type. To examine cell size, epidermal cells of sepal abaxial surfaces were observed using scanning electron microscope images (**Fig. 1E**). No significant change in *vaj-1* sepal cell length was observed (wild type, 44.67 ± 31.56 μm; *vaj-1*, 49.19 ± 46.78 μm; $P > 0.22$). In contrast, *vaj-1* sepal cell width was decreased to about 85% that of the wild type (wild type, 21.25 ± 8.22 μm; *vaj-1*, 18.09 ± 7.29 μm; $P < 0.001$). As the reduction of sepal cell width of *vaj-1* was about 15%, while the decrease in sepal width was about 35%,
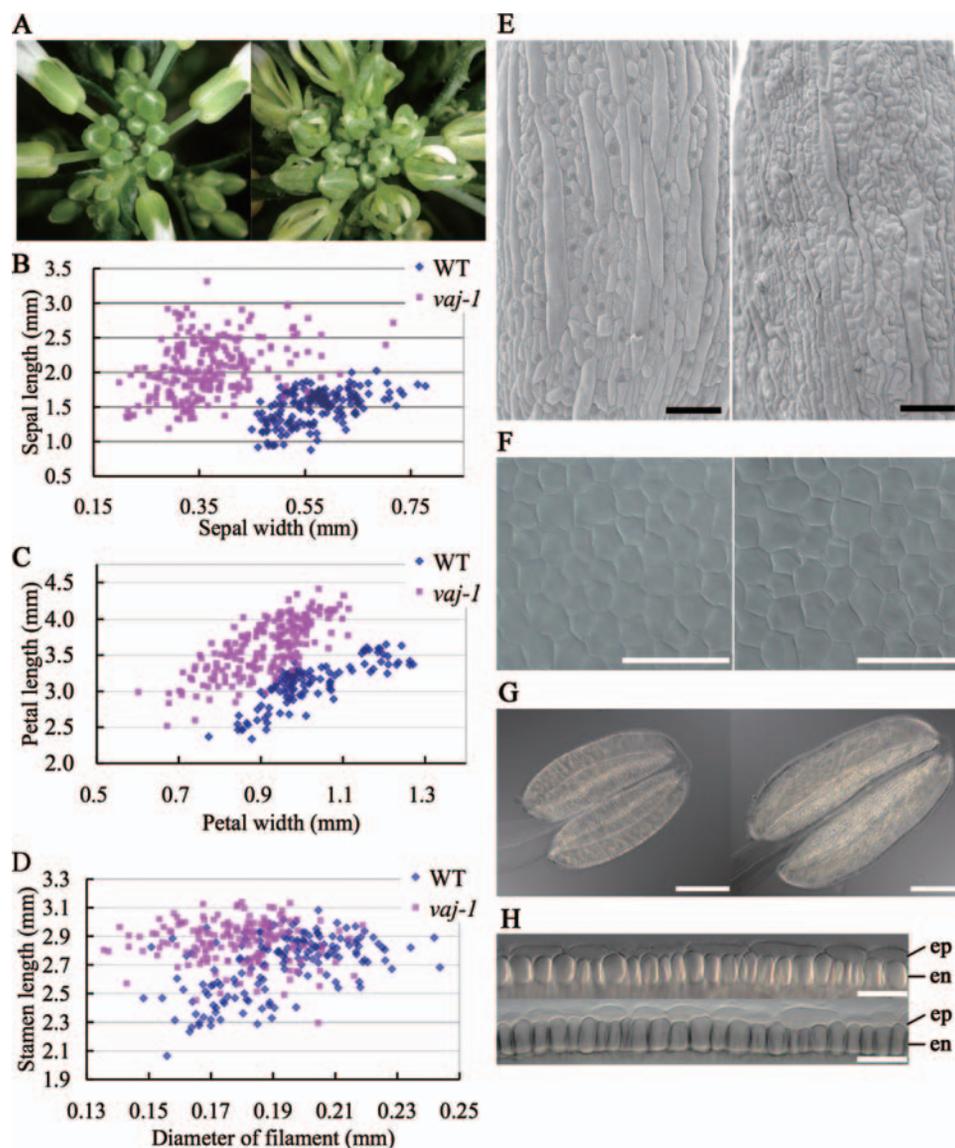

Fig. 1 vaj-1 mutation impairs floral organ development. (A) Inflorescences of the wild type (L*er*, left) and *vaj-1* (right). (B–D) The distribution of floral organ size in the wild type and *vaj-1*. In *vaj-1*, (B) sepals, (C) petals and (D) stamens grew to be narrower and longer than those of the wild type. (E) Scanning electron micrographs of sepal epidermal cells of the wild type (left) and *vaj-1* (right). Sepal epidermal cells of *vaj-1* were narrower than those of the wild type. Bars, 100 μm. (F) DIC images of cleared petal epidermal cells of the wild type (left) and *vaj-1* (right). Bars, 50 μm. (G) DIC images of cleared anthers of the wild type (left) and *vaj-1* (right). Bars, 200 μm. (H) Epidermal cells and endothecium cells of anthers of the wild type (top) and *vaj-1* (bottom). ep, epidermal cells; en, endothecium cells. Bars, 20 μm.

it was suggested that the defect in sepal width was a result of a decrease in both cell width and cell number along the sepal width. In turn, the defect in sepal length may be attributed to an increase in cell number along the sepal length.

We also analyzed the size of other floral organs in *vaj-1*. In *vaj-1* petals the width was decreased to about 90% (wild type, 1.02 ± 0.11 mm; *vaj-1*, 0.91 ± 0.10 mm; $P < 0.001$, **Fig. 1C**), and the length was increased up to about 1.2-fold (wild type, 3.10 ± 0.30 mm; *vaj-1*, 3.63 ± 0.37 mm; $P < 0.001$, **Fig. 1C**) when compared with those of the wild type. Analysis of cell size on the adaxial side of petals revealed that cell width and length were increased in *vaj-1* (**Fig. 1F**; cell width, wild type, 13.72 ± 1.96 µm; *vaj-1*, 16.58 ± 2.35 µm; $P < 0.001$; cell length, wild type, 15.65 ± 2.69 µm; *vaj-1*, 16.52 ± 2.88 µm; $P < 0.001$). These data indicated that in *vaj-1*, reduced petal width was due to a decrease in the cell number, and increased petal length was due to enlargement of petal cells. Stamen size was also affected in *vaj-1*. Stamen length including the anther and filament was increased (wild type, 2.70 ± 0.21 mm; *vaj-1*, 2.86 ± 0.14 mm; $P < 0.001$, **Fig. 1D**), filament width was decreased (wild type, 0.19 ± 0.02 mm; *vaj-1*, 0.18 ± 0.02 mm, $P < 0.001$, **Fig. 1D**) and anther length was about 1.2-fold greater than that of the wild type (wild type, 560.0 ± 31.7 µm; *vaj-1*, 681.1 ± 54.9 µm; $P < 0.001$, **Fig. 1G**). In contrast to the increase in anther length in *vaj-1*, the length of *vaj-1* anther endothecium cells was shorter than that of the wild type (wild type, 7.71 ± 1.87 µm; *vaj-1*, 7.43 ± 1.94 µm; $P < 0.001$, **Fig. 1H**). This indicated that the increase of anther length in *vaj-1* was a result of excess cell division. Taken together, these data suggest that during floral organ development *VAJ* is required both for the promotion of organ growth along the transverse axis and for the repression of growth along the longitudinal axis, and that both cell growth and cell proliferation are responsible for the control of directional organ growth.

In the wild-type flower, sepal primordia arise on the flanks of the floral meristem, and grow along the floral meristem (Smyth et al. 1990, **Fig. 2A**). At stages 3–4, *vaj-1* lateral sepal primordia appeared to be slightly smaller than those of the wild type (**Fig. 2A, E**), and at stages 5–6, lateral *vaj-1* sepal primordia were clearly smaller than those of the wild type (**Fig. 2B, C, F, G**). As a result, a large gap was formed between the lateral and neighboring sepals in *vaj-1* (**Fig. 2F, G, H**, arrowheads). This suggested that the *VAJ* gene is responsible for the proliferation of lateral sepal primordia. In addition, lateral sepal positions were sometimes shifted to the adaxial or abaxial sepals (**Fig. 2B, C, F, G**). In mature flowers, *vaj-1* sepals sometimes fused along their edge at the base (**Fig. 3A**, arrowheads). These observations raise the possibility that the shifts of sepal primordia could cause partial fusion of sepals in *vaj-1*. Fusion of floral organs was also found at the base of petals and stamens; however, it is not clear in these
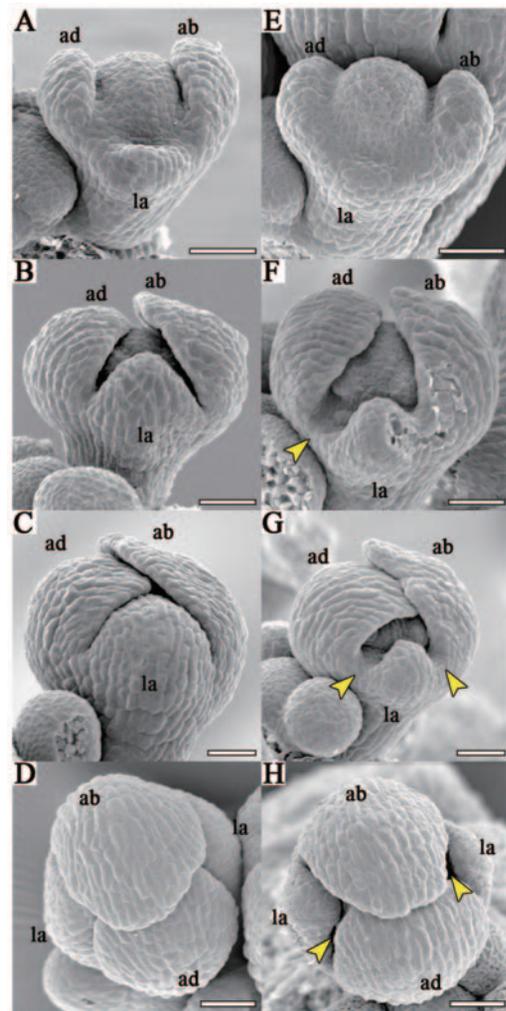

Fig. 2 *vaj-1* mutants show defects in sepal development from early floral stages. Scanning electron micrographs of early stage flowers from (A–D) the wild type and (E–H) *vaj-1*. At stages 3–4, lateral sepal primordia appeared to be slightly smaller than those of the wild type (A, E), and at stages 5–6 flowers of *vaj-1*, lateral sepal primordia are smaller than those of the wild type, producing space between two sepals (F, G, H, arrowheads). Images taken from the top of floral buds indicated that lateral sepal defects were prominent at early developmental stages (D, H). Numbers in figures indicate the developmental stages of flowers (Smyth et al. 1990). ad, adaxial; ab, abaxial; la, lateral sepals. Bars, 50 µm.

cases whether there were shifts in the position of petal and stamen primordia (**Fig. 3B, C**, arrowheads).

The *PRESSED FLOWER* (*PRS*) gene is known to be required for development of lateral sepals (Matsumoto and Okada 2001). To analyze the effect of the *vaj-1* mutation on *PRS* expression, we used the PRSp:GFP (green fluorescent protein) transgenic plant. At early floral stages, *PRS* expression was found in sepal margins of *vaj-1* (**Supplementary Fig. S1A–F**).

Fig. 3 vaj-1 mutation causes the fusion of floral organs. In vaj-1 flowers, floral organs were sometimes fused at the base (A, sepal; B, petal; C, stamens). Arrowheads indicate the region where organs were fused. Bars, 1 mm (A and C) and 0.5 mm (B).

Table 1 The number of floral organs was altered in vaj-1 mutant flowers

	No. of floral organs				
	2	3	4	5	6
Sepal					
Wild-type	0	0	100	0	0
vaj-1	4	13	73	9	1
Petal					
Wild-type	0	2	98	0	0
vaj-1	3	16	72	8	1
Stamen					
Wild-type			0	19	81
vaj-1			41	45	14
Carpel					
Wild-type	100	0			
vaj-1	88	12			

Values indicate the percentage of flowers harboring the indicated number of floral organs.
$n = 52$ (wild-type, Ler), $n = 92$ (vaj-1).

PRS was also expressed in petals of both wild-type and *vaj-1* flowers (**Supplementary Fig. S1G, I**). In addition, in *vaj-1* sepals, marginal cell files were observed (**Supplementary Fig. S1K, L**), but in *prs* mutants these cell files were absent.

In addition to changes in organ size, floral organ number was affected in *vaj-1* (**Table 1**). In *vaj-1*, the number of sepals and petals varied from two to six as compared with four in the wild type. Stamen number decreased from almost six to four or five, and carpel number slightly increased from two to three, in the wild type and *vaj-1*, respectively.

Molecular cloning of the VAJ gene

We cloned the *VAJ* gene using a map-based procedure. The *vaj-1* mutation was mapped to a position on chromosome 1 between marker 3 (M3) and marker 4 (M4) (**Fig. 4A**). Sequence analysis comparison of this region in *vaj-1* and the wild type showed a single base substitution, G to A, in the 5′ splice site of the second intron of At1g06220 (**Fig. 4B, C**). A complementation experiment using a genomic fragment containing the At1g06220 open reading frame (ORF) rescued the *vaj-1* phenotype (data not shown), indicating that the *VAJ* gene corresponds to At1g06220, previously described as *MEE5* (Pagnussat et al. 2005), *GFA1* (Coury et al. 2007) and *CLO* (Moll et al. 2008). Rapid amplification of cDNA ends (RACE) was performed using inflorescence cDNA, and the 5′- and 3′-untranslated region (UTR) of VAJ/GFA1/CLO mRNA sequences were 156 bp and 158 bp, respectively (**Supplementary Fig. S2**).

VAJ/GFA1/CLO protein showed similarity with human U5-116kD and yeast Snu114p (Coury et al. 2007, Moll et al. 2008). U5-116kD and Snu114p are U5 small nuclear ribonucleoprotein (snRNP)-specific proteins (Fabrizio et al. 1997, Achsel et al. 1998, Gottschalk et al. 1999, Stevens and Abelson 1999), and have a domain structure similar to ribosomal elongation factor EF-2, and also contain an N-terminal domain which is not present in EF-2 (Fabrizio et al. 1997,

Bartels et al. 2002). The N-terminal domain deletion mutant of yeast, snu114ΔN, shows a temperature-sensitive phenotype in growth and in pre-mRNA splicing (Bartels et al. 2002). This suggested that the unique N-terminal region distinguished the molecular function of U5-116kD and Snu114p from translation elongation factor EF-2. In VAJ/GFA1/CLO, this N-terminal domain was conserved (**Supplementary Fig. S3**). U5-116kD was shown to localize in the nucleus of HeLa cells (Fabrizio et al. 1997). To investigate the subcellular localization of VAJ/GFA1/CLO protein, we expressed VAJ/GFA1/CLO protein tagged with monomeric red fluorescent protein (mRFP) in *Arabidopsis* protoplasts. Transient expression analysis revealed that VAJ–mRFP was localized in the nucleus of the *Arabidopsis* protoplast, and exhibited a dot-like pattern (**Fig. 4E**). In plants, spliceosomal proteins are known to localize at speckles and Cajal bodies, which are the splicing factor compartments of the nucleus (Sleeman and Lamond 1999, Lorkovic and Barta 2004, Shaw and Brown 2004). Fluorescent-tagged *Arabidopsis* SR protein SC35–GFP was previously shown to localize mainly in the speckles of the nucleus (**Fig. 4F**, Lorkovic et al. 2004). Co-localization analysis using fluorescently tagged proteins showed that VAJ–mRFP co-localized with SC35–GFP, a speckle marker (**Fig. 4G**, Lorkovic et al. 2004). A similar result was reported showing that GFP–CLO co-localized with another nuclear speckle marker, SRp34–DsRED (*Discosoma* sp. red fluorescent protein) (Moll et al. 2008). These results suggested that VAJ/GFA1/CLO protein is a member of the U5 snRNP family.

Database searching identified homologs of VAJ/U5-116kD/Snu114p in various eukaryotic organisms, and the N-terminal region was shown to be conserved (**Supplementary Fig. S3**, Bartels et al. 2002). A phylogenic tree based
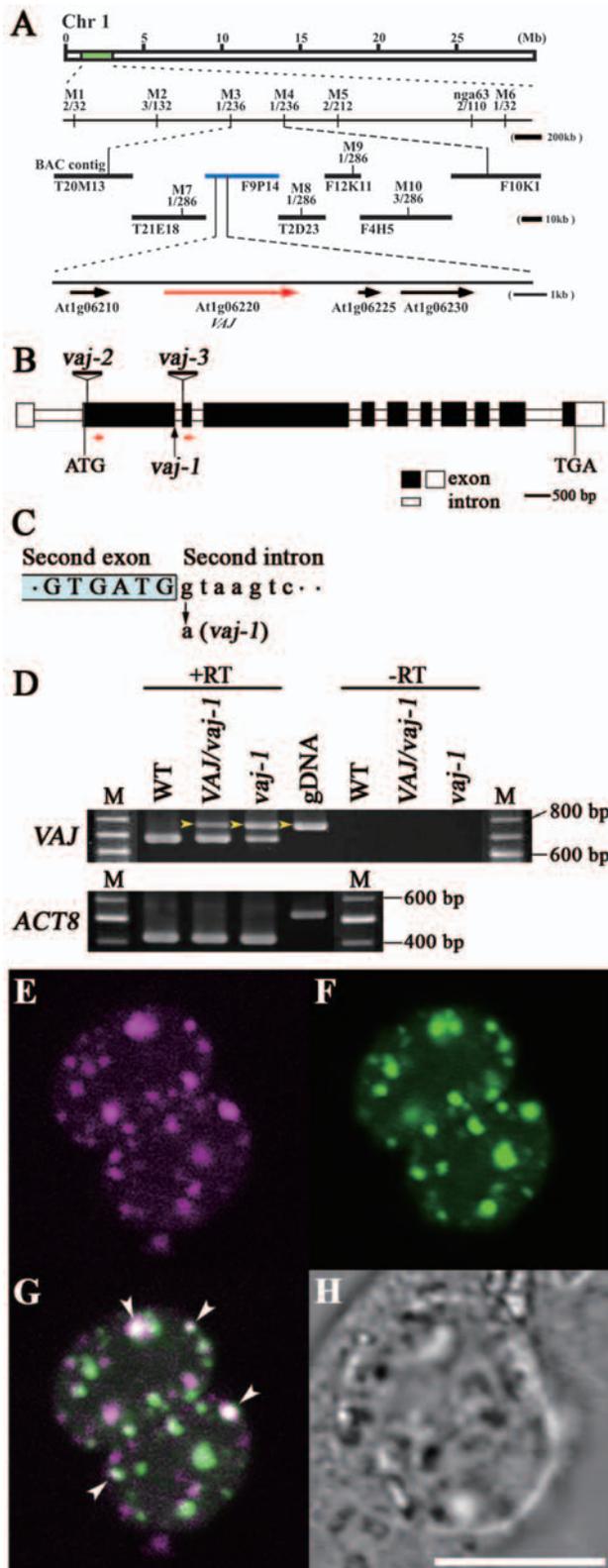
Fig. 4 Molecular cloning of the *VAJ* gene. (A) Chromosomal map position of the *VAJ* gene. The black bar represents BAC contigs around the *VAJ* locus. The polymorphism markers and numbers of

on the deduced amino acid sequences indicated that the homologs were clearly separated into three groups: plant, animal and fungi (**Supplementary Fig. S4**).

Expression pattern of the VAJ/GFA1/CLO gene during early floral organogenesis

To determine the location and timing of *VAJ/GFA1/CLO* expression in developing flowers, we performed mRNA in situ hybridization using inflorescence sections. *VAJ/GFA1/CLO* transcripts were detected throughout the inflorescence meristem and in young floral meristem through stage 3, in which high expression of *VAJ/GFA1/CLO* was found in the three layers (**Fig. 5A**). Four types of floral organs are mainly derived from these three layers of a floral meristem (Jenik and Irish 2000). At stage 6, *VAJ/GFA1/CLO* mRNA was also detected in sepals, and was highly expressed in developing organ primordia of petals, stamens and carpels (**Fig. 5B**), In stage 8 flowers, *VAJ/GFA1/CLO* expression is high in the interior of anther and the inner parts of carpel, where cell proliferation may be promoted to produce pollen and ovules. These findings indicated that *VAJ/GFA1/CLO* expression was induced in floral meristem prior to floral organ primordia formation and, after the induction, *VAJ/GFA1/CLO* mRNA continued to be expressed in developing floral organ primordia, suggesting that *VAJ/GFA1/CLO* is involved in the development of all types of floral organs from the initiation. We also generated transgenic plants expressing β-glucuronidase (GUS) under the control of the 2.3 kb *VAJ/GFA1/CLO* promoter. High levels of *VAJ/GFA1/CLO* expression were found in stamens at late floral stages 9–11

recombinants are indicated. Solid arrows are open reading frames. (B) Schematic diagram of the *VAJ* gene and location of the mutation site in *vaj-1* and T-DNA insertions in *vaj-2* and *vaj-3* alleles. The thick and thin boxes represent exons and introns, respectively. The white regions of the thick boxes correspond to UTRs. (C) The precise position of the *vaj-1* mutation is shown in (B). The *vaj-1* mutation converts the +1G of the 5′ splice site of the second intron in the *VAJ* gene. (D) RT–PCR assay using the gene-specific primers shown in (B) (red arrows) revealed that the *vaj-1* mutation partially abolished the *VAJ* pre-mRNA splicing in *VAJ/vaj-1* heterozygous plants and in *vaj-1* homozygous plants. Although a single band of PCR products was detected in the wild type, a second longer band PCR product was detected in *VAJ/vaj-1* and in *vaj-1* (arrowheads). Sequencing analysis revealed that the shorter band corresponded to PCR products originally from mature *VAJ* mRNA, suggesting that wild-type *VAJ* mRNA existed in *vaj-1*. +RT, with reverse transcriptase; –RT, without reverse transcriptase; M, markers. *ACT8* was used as a control. (E–H) Transient expression analysis of VAJ–mRFP and SC35–GFP in *Arabidopsis* protoplast. (E) VAJ–mRFP, (F) SC35–GFP, (G) a merged image of E and F, and (H) a corresponding differential interference contrast (DIC) image of E–G. Arrowheads indicate the position of VAJ–mRFP and SC35–GFP co-localization (white signal). Bar, 10 μm.
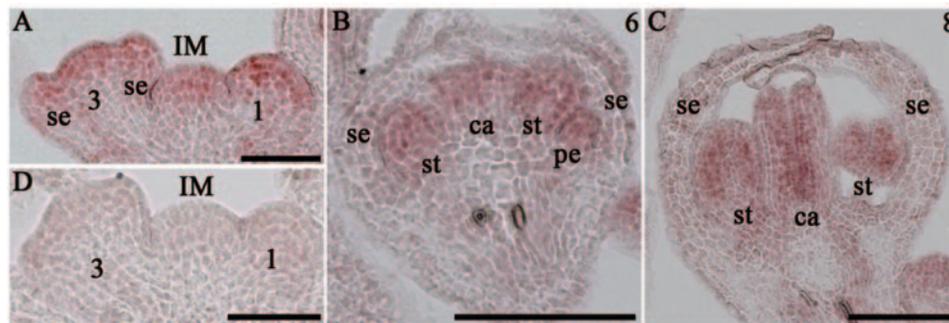
Fig. 5 Expression pattern of *VAJ* in early floral stages. In situ mRNA hybridization analysis of *VAJ* mRNA in wild-type inflorescence meristem and developing flowers. (A–C) Antisense probes, (D) sense probes. (A) *VAJ* mRNA was expressed throughout the inflorescence and flowers at stage 1 and stage 3. (B) A stage 6 flower. (C) A stage 8 flower. Strong *VAJ* expression was found in developing floral organ primordia. IM, inflorescence meristem; se, sepal; pe, petal; st, stamen; ca, carpel. Numbers in each photograph represent floral stages. Bars, 50 μm (A and D) and 100 μm (B and C).

(**Supplementary Fig. S5A**) and, consistent with a previous report, *VAJ/GFA1/CLO* expression was found in gynoecium and ovules at stages 12–13 (**Supplementary Fig. S5B, E**, Coury et al. 2007). Reverse transcription–PCR (RT–PCR) analysis revealed that *VAJ/GFA1/CLO* was expressed ubiquitously in the plant body (**Supplementary Fig. 5G, H**, Coury et al. 2007, Moll et al. 2008). GUS activity was found throughout the seedling; especially strong activity was found in the root tip including the meristematic region (**Supplementary Fig. S5C, D**).

vaj-1 mutation partially abolished VAJ pre-mRNA splicing

In *vaj-1*, a single base substitution was found in the splice site of the second intron. The *vaj-1* mutation converts the +1G of the *VAJ/GFA1/CLO* 5′ splice site in the second intron to A in the *vaj-1* allele (**Fig. 4B, C**). The plant intron 5′ splice site consensus sequence is AG/GTAAG, and many cases were reported in which mutations in this consensus sequence abolished splicing (Brown 1996). To determine whether *VAJ/GFA1/CLO* pre-mRNA was spliced normally in the *vaj-1* allele, RT–PCR assay using inflorescence mRNA was performed. Although in the wild type a single band of PCR products was detected, in the *vaj-1* allele two PCR product bands were detected (**Fig. 4D**). These two PCR products were purified and sequenced. The smaller band corresponded to PCR products produced from correctly spliced *VAJ/GFA1/CLO* mRNA in the wild type. The longer PCR product corresponded to PCR products produced from *VAJ/GFA1/CLO* pre-mRNA, and included the second intron (**Fig. 4D**, arrowhead). This splicing defect was also seen in *VAJ/vaj-1* heterozygous plants, which showed no phenotype in floral organs (**Fig. 4D**, arrowhead and **Supplementary Fig. S6A**). To determine the level of correctly spliced *VAJ/GFA1/ CLO* mRNA, we used semi-quantitative RT–PCR analysis. In *VAJ/vaj-1*, the level of mature *VAJ/GFA1/CLO* mRNA was about 80% of that of the wild type, and in *vaj-1* the level of

mature *VAJ/GFA1/CLO* mRNA was severely reduced to about 40% of that of the wild type (**Supplementary Fig. S6B**). These findings indicate that the +1G to A substitution blocked pre-mRNA splicing of the *VAJ/GFA1/CLO* gene, and suggested that the defects in floral organ growth in *vaj-1* might be related to the level of mature *VAJ/GFA1/CLO* mRNA. Because the mis-spliced *VAJ/GFA1/CLO* mRNA includes a premature stop codon in the fused sequence of the second intron, a truncated protein of 276 residues may be produced (**Supplementary Fig. S2**), lacking half of the G domain and the C-terminal portion containing domains II–V. The truncated VAJ/GFA1/CLO protein was predicted to be non-functional, because deletion of domain IV in Snu114p caused lethality in yeast (Bartels et al. 2003).

Gene expression profile in the inflorescence of the vaj-1 mutant

Nonsense-mediated decay (NMD) of mRNA is a well-conserved RNA surveillance system in eukaryotes, and serves to degrade mRNAs possessing premature translation termination codons (PTCs). In *Arabidopsis*, UPF family proteins, which have been found to function in the NMD system of other organisms, have been shown to be required for the degradation of aberrant mRNA containing PTCs (Hori and Watanabe 2005, Arciga-Reyes et al. 2006, Yoine et al. 2006). VAJ/GFA1/CLO protein is suggested to be involved in pre-mRNA splicing. Because mis-splicing in *vaj-1* may produce aberrant mRNAs harboring PTCs, mRNA expression levels of some genes would be changed in *vaj-1* mutants. To identify the genes whose expression level is changed in *vaj-1* mutants, microarray analysis was performed using the Agilent *Arabidopsis* 3 Oligo Microarray covering the entire genome. Total RNA was extracted from inflorescences excluding the opened flowers. Based on a 2-fold cut-off in the microarray data analysis, several genes whose expression level was significantly changed were identified (**Supplementary Tables S3, S4**). Functional classification of these differentially expressed

genes was conducted using The MIPS Functional Catalogue Database (FunCatDB) (http://mips.gsf.de/projects/funcat, Ruepp et al. 2004). As a result, it was shown that groups of differentially expressed genes between the wild type and *vaj-1* were restricted not only to the sets of genes involved in the developmental process, but the sets of genes required for various biological processes were also affected in the inflorescence of the *vaj-1* mutant (**Supplementary Table S5**).

Among the differentially expressed genes involved in the flower developmental process, *NAP* (*NAC-LIKE, ACTIVATED BY AP3/PI*) which was identified as a target gene of the floral homeotic genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Sablowski and Meyerowitz 1998), was found to be down-regulated in *vaj-1* mutants (**Supplementary Table S3**). Constitutive *NAP* expression driven by the 35S promoter inhibited cell expansion in petals and stamens, and antisense inhibition of *NAP* expression reduced cell elongation of stamens, suggesting that precise regulation of *NAP* expression timing is required for cell elongation (Sablowski and Meyerowitz 1998). To verify the microarray data, semi-quantitative RT–PCR analysis was carried out as described for detection of *NAP* expression, and showed that the *NAP* expression level was actually reduced (**Supplementary Fig. S7**). In contrast, no difference was found between the splicing pattern of *NAP* pre-mRNA in the wild type and *vaj-1*. These data suggested that the *NAP* expression level was directly or indirectly affected by the decrease in spliceosome activity caused by the *vaj-1* mutation.

The *vaj-1* mutation was shown to affect the transcripts level of genes involved in cell wall metabolism (**Supplementary Table S6**). About one-third of these gene products belonged to the glycosyl hydrolase family proteins, which may be involved in modification and reorganization of cell wall polysaccharides (reviewed by Minic and Jouanin 2006). Other affected cell wall-related genes encoded cell wall structural proteins (arabinogalactan proteins, hydroxyproline-rich proteins and glycine-rich proteins) and the cell wall loosening proteins the expansins.

Discussion

VAJ is required for directional control of floral organ growth

Polarized cell growth and cell proliferation affect organ shape and size. Many mutants and transgenic plants exhibit phenotypes of abnormal directional control of cell elongation and cell proliferation along the longitudinal and transverse axes in lateral organ morphogenesis (Tsuge et al. 1996, Kim et al. 2002, Narita et al. 2004, Y. K. Lee et al. 2006). However, such mutations affect the directional organ growth only in a single direction. In contrast, *vaj-1* mutants are unique because they exhibit different effects on floral organ growth in an axis-dependent manner. Previously, it was not clear

whether developmental controls along the longitudinal and transverse axes interacted. Because the *vaj-1* mutation demonstrated opposite effects on floral organ growth along the longitudinal and transverse axes, it seems possible that the *vaj-1* mutation affects an interaction between two axis-dependent controls of floral organ growth. However, it may be more plausible that the *vaj-1* mutation independently affected each directional organ growth axis, since VAJ/GFA1/CLO may function as a spliceosomal protein and the expression of many genes was altered in *vaj-1* flowers.

The *vaj-1* mutation diminished directional control of both cell proliferation and cell elongation. Microarray analysis revealed that there was no drastic change in the expression level of known genes involved in directional organ growth, such as *ROT4*, *AN*, *ROT3*, *LNG1* and *LNG2*, in the mutant. However, altered expression was observed for several XTH genes (**Supplementary Table S6**) encoding xyloglucan endotransglucosylase/hydrolase, which have a role in modification of the cellulose–xyloglucan network by splitting and reconnecting of xyloglucans (reviewed by Nishitani 1997, Rose et al. 2002). This suggested that the defects of polar cell elongation of sepals in *vaj-1* mutants may be attributed, to some extent, to the altered expression of the XTH genes.

Previous studies showed that several other mutations in *VAJ/GFA1/CLO* cause lethality during female gametophyte development, and no homozygous plant was obtained (Coury et al. 2007, Moll et al. 2008). In this study, we could isolate a new allele, *vaj-1*, whose mutation does not cause lethality because a certain level of *VAJ/GFA1/CLO* existed in *vaj-1* (**Fig. 4D**) and showed that *VAJ/GFA1/CLO* was required for the directional control of floral organ growth, which was not reported previously. In addition, it was also suggested that the depression of spliceosome activity was highly sensitized to the directional floral organ growth.

VAJ protein functions as a spliceosomal protein

VAJ/GFA1/CLO protein shows significant similarity with U5-116kD and Snu114p, which are U5 snRNP-specific proteins in human and yeast, respectively (Fabrizio et al. 1997, Achsel et al. 1998, Coury et al. 2007, Moll et al. 2008). Transient expression analysis using *Arabidopsis* protoplasts showed that VAJ–mRFP marked dot-like structures which coincided with those of SC35–GFP, a spliceosomal protein marker localized in the nucleus (**Fig. 4G**). This result was consistent with the co-localization of GFP–CLO and SRp34–DsRED (Moll et al. 2008). These observations suggested that VAJ/GFA1/CLO would function as a plant spliceosomal protein, an idea that is further supported by data described below.

Previous studies showed that *VAJ/GFA1/CLO* was required for female gametophyte development (Coury et al. 2007, Moll et al. 2008). In this study, we isolated T-DNA insertion lines from SIGnAL T-DNA insertion stocks (Alonso et al. 2003),

vaj-2 and *vaj-3*, which were not characterized in previous reports (**Supplementary Fig. S2**), and segregation analysis and reciprocal analysis revealed that *vaj-2* and *vaj-3* mutations also affected the female gametophyte (**Supplementary Tables S1, S2**). Cytological analysis of ovules of *vaj-2* and *vaj-3* heterozygous plants revealed that both mutations had the same effects on female gametophyte development, and unfertilized embryo sacs arrested at the different stages of megagametophyte development were found (**Supplementary Fig. S8**). Analysis of seed set in dry mature siliques of *VAJ/vaj-2* and *VAJ/vaj-3* indicated that *vaj-2* and *vaj-3* mutations might affect embryogenesis as well as female gametophyte development (**Supplementary Fig. S9**).

Three other genes encoding spliceosomal proteins were reported to affect female gametophyte development and embryogenesis when mutated: *LACHESIS* (*LIS*), *ATROPOS* (*ATO*) and *SUS2*. *LIS* and *ATO* were isolated as genes involved in female gametophyte development (Gross-Hardt et al. 2007, Moll et al. 2008), and encode proteins homologous to Prp4p and SF3a60, respectively. Prp4p is a U4/U6 snRNP-specific protein (Banrcoques and Abelson 1989, Bjorn et al. 1989). SF3a60 is a subunit of splicing factor 3a, required for the formation of the mature 17S U2 snRNP (Abovich et al. 1990, Nesic and Kramer 2001). The *SUS2* gene was shown to encode a protein homologous to Prp8p/U5-220kD in *Arabidopsis*, and *sus2* mutants exhibit an embryo-defective phenotype with an enlarged suspensor (Schwartz et al. 1994, Meinke 1996). These observations indicated that the functional spliceosome is essential for female gametophyte and embryo development.

STABILIZED1 (STA1) protein, which is required for the response to cold stress and abiotic stresses, shows high sequence similarity with human U5-102kD, yeast prp1+ (fission yeast) and Prp6p (budding yeast) (B. H. Lee et al. 2006). Microarray analysis revealed that two genes encoding spliceosomal proteins were highly expressed in *sta1* mutants (B. H. Lee et al. 2006). Lee et al. suggested that the up-regulation of the expression level of these two genes might be due to the compensation system working for the mRNA splicing defects caused by the *sta1* mutation. Interestingly, one of the up-regulated genes, At1g28060, was also up-regulated in *vaj-1* (fold change, 1.60 ± 0.06). In addition to this increase, we found that the decrease in the expression level of atRSZ33, which is a plant-specific SR protein involved in spliceosome assembly (Lopato et al. 2002), was found in *vaj-1* (fold change, 0.61 ± 0.01). These data suggested that the compensation system would also function in the *vaj-1* mutant.

Although the expression of *VAJ/GFA1/CLO* was found in almost all parts of plants (**Fig. 5H**, Coury et al. 2007, Moll et al. 2008) and the protein function is essential in pre-mRNA splicing, *vaj-1* showed phenotypes restricted to floral organ growth. This suggested that floral organ development might be more susceptible to the depression in the activity of the spliceosome. A similar phenomenon was known in the case of a human ophthalmic hereditary disorder, retinitis pigmentosa. This disease is caused by retinal degeneration with commencement of loss of photoreceptor cells. Four out of 12 autosomal dominant retinitis pigmentosa (adRP) genes (*PRPF3*, *PRPF8*, *PRPF31* and *PAP-1*) are ubiquitously expressed genes encoding proteins required for proper assembly and function of the U4/U6/U5 tri-snRNP complex (Kennan et al. 2005, Mordes et al. 2006, Wang and Cooper 2007). These mutations impairing apparently basic function in pre-mRNA splicing only affect a specific type of neurons. Several models were proposed to explain the photoreceptor-specific phenotype. Outer segment discs enriched with rhodopsin in photoreceptors undergo continuous renewal every 10 d, which produces high demands for mRNA expression and protein synthesis. The four mutations in adRP genes encoding spliceosomal proteins may decrease the spliceosome activity, and pre-mRNA splicing would be a rate-limiting step in photoreceptor cells but not in other cell types. Alternatively, dominant negative effects of mutations on spliceosomal protein function might be considered to lead to cell death of photoreceptor cells.

In this study, we found that the single base substitution in the splice site of the second intron of *VAJ/GFA1/CLO* abolished *VAJ/GFA1/CLO* pre-mRNA splicing (**Fig. 4D**). RT–PCR analysis revealed that compared with the wild type the expression level of mature *VAJ/GFA1/CLO* mRNA was reduced to about 80 and 40% in *VAJ/vaj-1* heterozygous plants and *vaj-1* homozygous plants, respectively (**Supplementary Fig. S6B**). A moderately reduced level of *VAJ/GFA1/CLO* mRNA in *VAJ/vaj-1* caused no phenotype in floral organ development (**Supplementary Fig. S6A**). This indicated that the expression level of mature *VAJ/GFA1/CLO* mRNA in *vaj-1* dropped below the threshold required for proper floral organ development. It might be possible that some genes involved in the directional control of floral organ growth demand higher activity of the spliceosome than other genes.

Given that there is no difference in transcriptional efficiency between wild-type *VAJ/GFA1/CLO* pre-mRNA and mutant pre-mRNA harboring the *vaj-1* mutation, enrichment of incorrectly spliced *VAJ/GFA1/CLO* mRNA in *vaj-1* would be twice that in *VAJ/vaj-1*. However, the relative amount of mis-spliced *VAJ/GFA1/CLO* mRNA in *vaj-1* was more than twice as great as that in *VAJ/vaj-1* (**Supplementary Fig. S6B**). It would be plausible that not only the base substitution in the 5′ splice site of intron but also the decrease of spliceosome activity due to the *vaj-1* mutation would affect pre-mRNA splicing of *VAJ/GFA1/CLO*.

Materials and Methods

Plant material and growth conditions

vaj-1 was isolated from an M_2 population of ethylmethane sulfonate-mutagenized seeds of *Arabidopsis* Ler. *vaj-2* and *vaj-3* alleles, SALK_143877 and SALK_025707, respectively, were SALK T-DNA insertion lines provided by the ABRC (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.html) (Alonso et al. 2003). To confirm the T-DNA insertion sites, the following primer sets were used: 5′-GCGTGGACCGCTTGCTGCAACT-3′(LBb1)and5′-AACTCC TAAGTGACATACGAC-3′ for LB in *vaj-2*; LBb1 and 5′-GCT CATCGACTCGTGTGTCTG-3′ for RB in *vaj-2*; 5′-AGCTGTT GCCCGTCTCACTGGTG-3′ (PL13) and 5′-CTCAGCTTGTAG TAAGCATCC-3′ for LB in *vaj-3*; and PL13 and 5′-ATGCT GCTGAAGGAGTGATGG-3′ for RB in *vaj-3*. Ler and Columbia were used as controls. Seeds were surface sterilized and sown on soil or on a Murashige and Skoog (MS) agar plate (Invitrogen, Carlsbad, CA, USA). Seeds were incubated for 3 –4 d in the dark at 4°C for stratification. Plants were grown under continuous white fluorescent light at 22°C.

Phenotypic analyses of vaj-1

Flowers and floral organs were visualized with a Leica M420 macroscope (Leica, Wetzlar, Germany). Images were taken with a Coolpix 990 digital camera (Nikon, Tokyo, Japan). For measurements of sepal sizes, images taken from the adaxial side of dissected sepals placed on agar plates were used. Petals were smoothed out and affixed to double-faced tape. Stamens were fixed overnight in an ethanol : acetic acid solution (9 : 1) at room temperature, dehydrated with an ethanol solution series (90, 70, 50, 30 and 10%, each step 20 min) and mounted with clearing reagents, choral hydrate : glycerol : water (8 : 1 : 2, w:v:v). Cleared stamens were observed with differential interference contrast (DIC) optics on an AxioPlan2 microscope (Carl Zeiss, Jena, Thuringia, Germany), and images were taken with a AxioCam HR (Carl Zeiss, Jena, Thuringia, Germany). For observation of sepal epidermal cells, scanning electron microscopy was performed as described previously (Matsumoto and Okada 2001). Petal cells were visualized by clearing with clearing reagents as described above. Images were analyzed by Image Pro Plus software (Media Cybergenetics, Silver Spring, MD, USA).

Molecular cloning of the VAJ gene

vaj-1 plants were crossed with Columbia ecotype, wild-type plants. A total of 118 F_2 plants showing the narrow sepal phenotype were identified. Initial mapping using these F_2 plants located *VAJ* between marker M3 on bacterial artificial chromosome (BAC) T20M13 (TAIR accession No. 49614) and marker M4 on BAC F10K1 (accession No. AC067971) of chromosome 1. Using M3 and M4 markers, >15 recombinants

were obtained from 798 F_2 plants. Additional mapping using these recombinants revealed that the *VAJ* gene was located in the region between markers M7 and M8. All predicted ORFs in this region were sequenced in Ler and *vaj-1*, and a point mutation was found in At1g06220. Primer sequences used for mapping markers are shown in **Supplementary Table S5**.

For the complementation test, a genomic fragment including the At1g06220 ORF (corresponding to bp 23,213–31,081 of BAC F9P14; accession No. AC025290) was subcloned into pDONR/Zeo (Invitrogen, Carlsbad, CA, USA), and the genomic fragment was transferred into pPZP211N-PGW (generated by Dr. Taisuke Nishimura) by LR reaction. Transformation of the genomic fragment into *vaj-1* was performed by vacuum infiltration using *Agrobacterium tumefaciens*.

For determination of 5′-UTR and 3′-UTR sequences of *VAJ*, RACE was performed using a BD SMART RACE cDNA amplification kit (Clontech , Palo Alto, CA, USA). The gene-specific primer sequences used are shown here: VAJ-5GSP, 5′-AGGCCCTTGTTCGCCATCGGAACC-3′; VAJ-5NGSP, 5′-CTGTCAGACTCAATCTCAGGTCCAAC-3′; VAJ-3GSP, 5′-GCCATCCAGTTGCGCCCACTTGAG-3′; and VAJ-3NGSP, 5′-CGCCGTAGAAAGGGAATGAGCGAAGAC-3′

Expression analyses of VAJ

Total RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA), and cDNA was synthesized using a SuperScriptIII First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). To check the splicing pattern of the *VAJ* mRNA, gene-specific primers 5′-AAGAAAATGGTTCCGAT GGCG-3′ (primer A) and 5′-ACAATAGGGAGATGGT CCTGG-3′ were used. For the detection of *VAJ* mRNA, the expression primer A shown above and 5′-CTCAGCTTGT AGTAAGCATCC-3′ were used. To detect *ACT8* expression, primers 5′-ATGAAGATTAAGGTCGTGGCA-3′ and 5′-TCCG AGTTTGAAGAGGCTAC-3′ were used, as described previously (Aida et al. 1997).

In situ mRNA hybridization was performed as described previously (Ueda et al. 2004). Inflorescences of Ler ecotype plants were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde). A portion of *VAJ* cDNA was amplified by PCR with primers 5′-CTTGAATTCTGAGTTTGGTAACTA TGTTGG-3′ and 5′-CAGGAATTCGGTAGTACTTCTTATC CTCAG-3′, and the PCR product was subcloned into pBlue-Script SK II (Stratagene, La Jolla, CA, USA). Preparation of labeled probes was performed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA).

To generate the *VAJ* promoter–GUS construct, the *VAJ* promoter region (corresponding to bp 23,200–25,532 of BAC clone F9P14) was amplified by PCR and inserted between the *Hin*dIII and *Sal*I sites of the pBI101.1 binary

vector (Jefferson et al. 1987). The construct was transformed into the Columbia ecotype by vacuum infiltration. For the detection of GUS activity and the visualization of GUS staining patterns, a previously described method was used (Donnelly et al. 1999).

Transient expression analysis

The method for transient expression in *Arabidopsis* Col-0 suspension culture cells was described previously (Ueda et al. 2001). Transformed protoplasts were observed with a confocal laser scanning microscope (LSM5 Pascal, Carl Zeiss, Jena, Thuringia, Germany). The coding region of *VAJ* cDNA excluding the stop codon was amplified with primers, 5′-CA CCTCGAGAATAAACCATGGAAAGTAGCTTGTATGAC-GAG-3′ and 5′-CAGAGAGGATCCAATCATCTGCAGATGG AGATCGCC-3′, and the PCR product was inserted between the *Sal*I and *Bam*HI sites of pDEDH-mRFP (Lorkovic et al. 2004), upstream of the mRFP tag.

Cytological analysis of embryo sacs

Pistils of self-pollinated flowers of *vaj-2* and *vaj-3* heterozygous plants were used for cytological analysis of embryo sacs. Fixation and clearing of embryo sacs and developing embryos were performed by the methods described previously (Yadegari et al. 1994). Dissected immature seeds were examined using an Axioplant2 microscope (Carl Zeiss, Jena, Thuringia, Germany), and images were taken with an AxioCam HR digital camera.

Microarray analysis

Total RNA isolated from inflorescences of the wild type, L*er*, and *vaj-1* (two independent replications) were used for the preparation of Cy3- and Cy5-labeled cRNA probes with a Low RNA Fluorescent Linear Amplification Kit (Agilent Technologies Inc., Wilmington, DE, USA). Microarray experiments were performed with the Agilent *Arabidopsis* 3 Oligo Microarray (Agilent Technologies Inc., Wilmington, DE, USA) and an Agilent Microarray Scanner (model G2505B; Agilent Technologies Inc., Wilmington, DE, USA).

Supplementary data

Supplementary data are available at PCP online.

Funding

Acknowledgments

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