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 primordia 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,
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, LONGIFOLIA1 (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 (Szesci 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 (Szesci 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, Snu7 (Clay and Nelson 2005). The SMP1 gene was shown to be highly expressed in the region of cell proliferation. Accordingly, in the smp5 deletion mutants, 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 smp5 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 produces 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 US-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 (Ler). 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%,
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 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 *PRS::GFP* (green fluorescent protein) transgenic plant. At early floral stages, *PRS* expression was found in sepal margins of vaj-1 (Supplementary Fig. S1A–F).
**Table 1** The number of floral organs was altered in vaj-1 mutant flowers

<table>
<thead>
<tr>
<th>No. of floral organs</th>
<th>Sepal</th>
<th>Petal</th>
<th>Stamen</th>
<th>Flower</th>
<th>Carpels</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>13</td>
<td>73</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>2</td>
<td>98</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>16</td>
<td>72</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>41</td>
<td>45</td>
<td>14</td>
</tr>
</tbody>
</table>

Values indicate the percentage of flowers harboring the indicated number of floral organs. n = 52 (wild-type, L), 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 sepal and petal cells 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 sepal and petals varied from two to six 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′-end of the second intron of At1g06220 (Fig. 4B). We cloned the molecular cloning of 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′-end of the second intron of At1g06220 (Fig. 4B). We cloned the

**Axis-dependent regulation of floral organ growth**

**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, stamen). Arrowheads indicate the region where organs were fused. Bars, 1 mm (A and C) and 0.5 mm (B).
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.
(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. S5G, 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/funct, 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 antipsis 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 sepal 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 (Banroques and Abelson 1989, Bjorn et al. 1989). SF3a60 is a subunit of splicing factor 3a, required for the formation of the mature 175 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, Atg28060, 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/US 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.
Molecular cloning of the VAJ gene

vaj-1 plants were crossed with Columbia ecotype, wild-type plants. A total of 118 F2 plants showing the narrow sepal phenotype were identified. Initial mapping using these F2 plants located VAJ between marker M3 on bacterial artificial chromosome (BAC) T2OM13 (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 F2 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 cloned into pDONR/Zeo (Invitrogen, Carlsbad, CA, USA), and the genomic fragment was transferred into pPGW (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′-AGGCCCTTGTTCGCCATCGGAAAC-3′; VAJ-3NGSP, 5′-CTTCAGACTCAATCTCAGGTCCA-3′; VAJ-3GSP, 5′-GCATGATTGCGCCCCACTTGAG-3′; and VAJ-3NGSP, 5′-CGCCTAGAAAGGGAATTAGCAGGACGAC-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 SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). To check the splicing pattern of the VAJ mRNA, gene-specific primers 5′-AAGAAAAATGTGTGGTGGCCATCGGAAAC-3′; and VAJ-3NGSP, 5′-CTTCAGACTCAATCTCAGGTCCA-3′; VAJ-3GSP, 5′-GCATGATTGCGCCCCACTTGAG-3′; and VAJ-3NGSP, 5′-CGCCTAGAAAGGGAATTAGCAGGACGAC-3′.

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′-GTGATGGATGGATGGTCTCAGTTGTAG-3′ and 5′-ACTCCAGGCATGATTGCGCCCCACTTGAG-3′; and 5′-CTCAGCTCAGTGCCAGGAC-3′ and 5′-TCCGAGTTTGAGAGGAGCTAC-3′, which were used to detect VAJ expression. The expression primer A shown above and 5′-UTR sequences of Ler and vaj-1 were used for mapping markers are shown in Supplementary Table S5.
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, S’-CA CCTGAGAATAAAACATGGAAGTAGCTTTGTAGAAGAG-3’ and S’-CAGAGGATCCATCTGCAAGTAGGAGATGCCC-3’, and the PCR product was inserted between the SalI and BamHI 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, Ler, 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.

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**References**


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