Plant Biotechnology 29, 1–8 (2012)
DOI: 10.5511/plantbiotechnology.11.1101a

Original Paper

Roles of the *ASYMMETRIC LEAVES2* gene in floral organ development in *Arabidopsis thaliana*

Sumie Keta[1], Hidekazu Iwakawa[2,a], Masaya Ikezaki[3], Endang Semiarti[4], Shoko Kojima[1], Yasunori Machida[3], Chiyoko Machida[1,2,*]

[1] Graduate School of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi 487-8501, Japan; [2] Plant Biology Research Center, Chubu University, Kasugai, Aichi 487-8501, Japan; [3] Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Aichi 464-8602, Japan; [4] Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia
* E-mail: cmachida@isc.chubu.ac.jp Tel & Fax: +81-568-51-6276

Received August 25, 2011; accepted November 1, 2011 (Edited by T. Mizoguchi)

Abstract The *ASYMMETRIC LEAVES2* (*AS2*) gene in *Arabidopsis thaliana*, a member of the *AS2/LOB* gene family, is involved in the development of a symmetrical expanded lamina, with the rachis as the axis. On flowering, transcripts of *AS2* were detected in inflorescence meristems, floral meristems, and primordia of all floral organs. Levels of accumulation of *AS2* transcripts were then decreased in the late stage of the floral organ primordia. *β*-glucuronidase (GUS) activities were examined in transgenic plants that had been transformed with the GUS gene controlled by the *AS2* promoter and were detected in early stages of flower development. We quantified the length of floral organs at each developmental stage. Our results showed that the ratios of lengths of stamen to sepal and carpel to sepal in the *as2-1* and *as1-1* mutants were larger than those seen in wild-type plants in stages 12 to 14. Furthermore, the changes in the ratios of the lengths of floral organs in *as2-1* and *as1-1* mutants were suppressed by the mutations of class 1 *KNOX* genes. These results indicate that *AS2* and *AS1* genes would control the balance of the floral organ lengths via repressing class 1 *KNOX* genes in reproductive stages.

Key words: *ASYMMETRIC LEAVES1* (*AS1*), AS2/LOB family, class 1 *KNOX*, floral meristem, proximal-distal axis.

The *ASYMMETRIC LEAVES2* (*AS2*) gene, a member of the *AS2/LOB* gene family, and the *ASYMMETRIC LEAVES1* (*AS1*) gene, which encodes a myb transcription factor, are involved in the development of a symmetrical expanded lamina, with the rachis as the axis in *Arabidopsis thaliana* (Byrne et al. 2000; Byrne et al. 2002; Iwakawa et al. 2002; Semiarti et al. 2001). *AS2* and *AS1* repress the class 1 *KNOTTED*-like homeobox (*KNOX*) genes, *BREVIPEDICELLUS* (*BP*)/*KNAT1*, *KNAT2*, and *KNAT6*, for maintenance of the determinate state of cells in leaves. In addition to class 1 *KNOX* genes, *AS2* and *AS1* repress the *ETTIN*/*AUXIN RESPONSE FACTOR3* (*ARF3*), *KANADI2*, and *YABBY5* genes, which are abaxial determinants, indicating that *AS2* and *AS1* are involved in specification of adaxial cell fate determination in leaves (Byrne et al. 2000; Iwakawa et al. 2007; Ori et al. 2000; Semiarti et al. 2001; Takahashi et al. 2008). It was shown that AS1 binds to the *BP* and *KNAT2* promoter and directly represses the expression of these genes during leaf development (Guo et al. 2008). These observations suggest that *AS2* and *AS1* are multiple transcriptional regulators, although the molecular mechanisms of multiple repression have not been clarified. The functions of *AS2* and *AS1* in the vegetative stage are well studied (Byrne et al. 2000; Byrne et al. 2001; Byrne et al. 2002; Ikezaki et al. 2010; Iwakawa et al. 2002; Iwakawa et al. 2007; Kojima et al. 2011; Matsumura et al. 2009; Ori et al. 2000; Rédei and Hirono 1964; Semiarti et al. 2001; Takahashi et al. 2008; Tsukaya and Uchimiya 1997; Ueno et al. 2007), however, little is known about their functions in the reproductive stage.

On flowering, *AS1* transcripts are detected on the flank of the inflorescence apex in a region corresponding to the cryptic bract (Byrne et al. 2000). In early floral primordia, *AS1* expression is first detected in sepal primordia and subsequently in primordia of all floral organs (Byrne et al. 2000). Although *AS2* transcripts have been detected in flower buds by northern blot analysis (Iwakawa et al. 2002), the accumulation pattern has not yet been reported. In the present study, we analyzed the expression patterns of *AS2* in floral organs by *in situ* hybridization and the GUS reporter gene, and show

Abbreviations: GUS, *β*-glucuronidase; MS, Murashige and Skoog

[a] Present address: Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392, U.S.A.

This article can be found at http://www.jspcmb.jp/
Published online February 20, 2012

that the *AS2* gene functions in the development of floral organs.

There have been several reports about floral morphology concerned with the *AS2* and *AS1* genes. In the flowers of *as2-1* mutants, it is reported that the relatively narrow sepals and the petals are unusually curled downwardly, but are not strongly asymmetric (Semiarti et al. 2001). The sepals and petals in *as2-1* mutants show less complex venation than that seen in the wild type (Semiarti et al. 2001). The sepals and petals of both *as2-1* and *as1-1* mutants are shorter than those of the wild type (Byrne et al. 2002; Ikezaki et al. 2010), and this phenotype is suppressed by a triple mutation of three class 1 *KNOX* genes, *BP*, *KNAT2*, and *KNAT6*, suggesting that *AS2* and *AS1* are involved in the formation of sepals and petals through repression of the class 1 *KNOX* genes (Ikezaki et al. 2010). Recently, it has been reported that *AUXIN RESPONSE FACTOR6* (*ARF6*) and *ARF8* repress class 1 *KNOX* genes in floral organs in parallel with *AS2* and *AS1* (Tabata et al. 2010). However, quantitative analysis in the development of floral organs involved in *AS2* and *AS1* has not yet been reported. In this study, we measured the lengths of floral organs at each developmental stage. Our results suggested that *AS2* and *AS1* controlled the morphology of floral organs with respect to the shape and balance of the length of each floral organ. Furthermore, the balance of the length of floral organs might be regulated through the repression of class 1 *KNOX* genes.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 (CS1092) and mutants *as2-1* (CS3117) and *as1-1* (CS3374) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, USA). We outcrossed *as2-1* with Col-0 three times and *as1-1* with Col-0 once and used the progeny for our experiments (Semiarti et al. 2001). The *bp-1 knat2-3 knat6-2* mutant was described in Ikezaki et al. (2010). For the analysis of plants, seeds were sown on soil or on Murashige and Skoog (MS) medium. After 2 days at 4°C in darkness, plants were transferred to a regimen of white light at 50 μmol m^{-2} S^{-1} for 16 h daily at 22°C, as described previously (Semiarti et al. 2001).

In situ hybridization

For *in situ* hybridization, tissues were fixed, dehydrated, and mounted in Paraplast plus (Sigma-Aldrich, Tokyo, Japan) essentially as described previously (Long and Barton 1998; Semiarti et al. 2001; Tanaka et al. 2002). Sections (8 μm thickness) were cut with a microtome (Leica Microsystems, Wetzlar, Germany). Antisense and sense RNA probes for *AS2* were generated as described previously (Iwakawa et al. 2002). The plasmid pAS1c was constructed by inserting a fragment of *AS1* cDNA, which had been extended from the *Stu*I site to the termination codon, between the *Hinc*II and *Not*I sites of pBluescriptII SK(-). An antisense RNA probe for *AS1* was generated by T3 RNA polymerase after linearization of plasmid pAS1c with *Cla*I. A sense RNA probe for *AS1* was generated by T7 RNA polymerase after linearization of pAS1c with *Pst*I. The visualization reaction was performed for 2 or 3 days.

Histochemical assay for GUS activity

Five plants contained pAS2 : GUS. Histochemical detection of GUS activity was performed essentially as described by Koizumi et al. (2000). Fixed plants were incubated in GUS staining solution [100 mM sodium phosphate, pH 7.2, 1 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100, and 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc)] at 37°C for 24 h.

Results

Analysis of AS2 gene expression patterns in the reproductive stage by in situ hybridization

To ascertain the function of *AS2* in the reproductive stage, we first investigated the expression patterns of *AS2* by *in situ* hybridization. Transcripts of *AS2* were detected in inflorescence meristems, floral meristems, and primordia of floral organs (Figure 1A, B). Signals in the peripheral zone were detected, but no clear signal was detected in the central zone of the inflorescence meristem (Figure 1A). Signals in the distal and lateral parts of organ primordia of the floral organs were stronger than those in the proximal and medial parts of the primordia at stages 2 to 5 (Figure 1A, B). Signals were then decreased in the later stages of floral organ primordia (Figure 1B). In the late stage of floral organ primordia, signals were detected in cells in the stamen primordia and the gynoecium primordium (Figure 1C). No signals were detected in the *AS2* sense control (Figure 1D).

Similarly as for *AS2*, we also investigated the expression pattern of *AS1*. We observed signals in inflorescence meristems, floral meristems, and all floral organs, as described previously by Byrne et al. (2000) (Figure 1E, F). Signals of *AS1* transcripts were decreased in later stages of primordia similarly as seen in the accumulation of *AS2* transcripts (Figure 1E).

The cumulated transcripts of both *AS2* and *AS1* genes were detected in the floral meristems and floral organ primordia in the reproductive stage. These results supported the idea that *AS2* and *AS1* might function together in both the reproductive stage as well as the vegetative stage.

Analysis with a reporter gene for β-glucuronidase (GUS)

For further detailed analysis of the expression pattern of *AS2*, we examined GUS activity in transgenic plants that had been transformed with the GUS gene controlled by the *AS2* promoter. We used plants with the *AS2*-fused GUS construct (pAS2 : GUS), in which the DNA sequence that encoded GUS was fused to the 3301 bp sequence upstream of the *AS2* gene and 18 bp of the N-terminal *AS2* coding region (Iwakawa et al. 2007). We have shown previously that the above-described *AS2* genomic sequence encompasses the *AS2* promoter (Iwakawa et al. 2002). In addition, the 18 bp of coding sequence was essential for expression of the GUS fusion gene (Iwakawa et al. 2007). We obtained five independent transgenic lines that harbored pAS2 : GUS, and all of them yielded essentially the same expression pattern of GUS activity. As shown in Figure 2, the GUS activity in pAS2 : GUS plants was detected in the early stage of flowers. Signals of the stamen were stronger than those of other floral organs (Figure 2A–C, E–G). Signals were then decreased in the later stages similarly as in the cumulation of transcripts seen by *in situ* hybridization (Figure 2D, H).

These expression patterns were consistent with the patterns in flower buds obtained by *in situ* hybridization (Figure 1A, B). These results indicate that *AS2* functions in the development of floral organs.

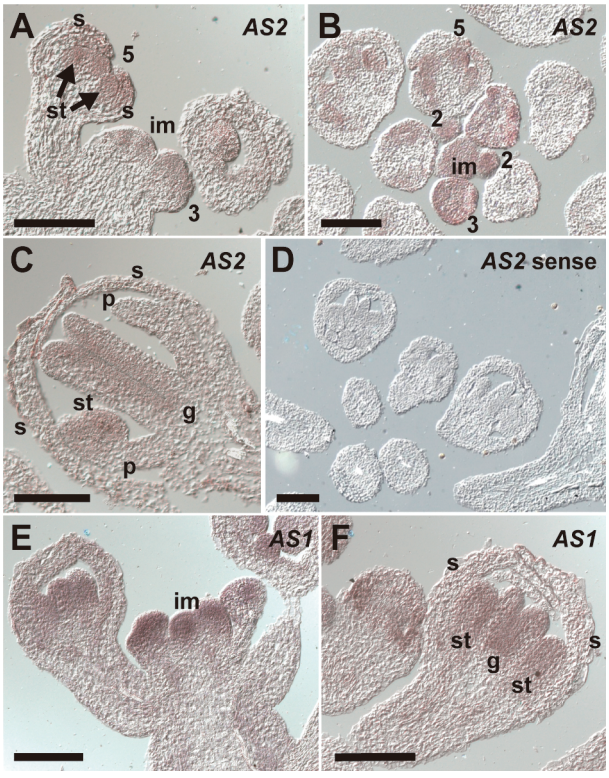

Figure 1. Expression pattern of *AS2* and *AS1* in reproductive stage. (A–C) Patterns of distribution of *AS2* transcripts obtained with *AS2* antisense probe. (D) Sense control for A, B and C. (E, F) Patterns of distribution of *AS1* transcripts obtained with *AS1* antisense probe. Numbers show the stage of flowers. (im) inflorescence meristem, (s) sepal, (p) petal, (st) stamen, (g) gynoecium. Scale bars: 50 μm.

Phenotype of floral organs in the as2 and as1 mutants

The flowers of *as2-1* and *as1-1* mutants showed the characteristic phenotype of protruding stigmas. It has been reported that the sepals and petals of *as2-1* and *as1-1* are shorter than those of the wild type (Byrne et al. 2002; Ikezaki et al. 2010). To ascertain the effect of *AS2* and *AS1* mutations on the phenotypes of reproductive organs precisely, we quantified the lengths of sepals, petals, stamens, and carpel at each developmental stage in these mutants. We followed the definition of flower developmental stages according to Smyth et al. (1990). At stage 13 in the wild-type plants, petals seen

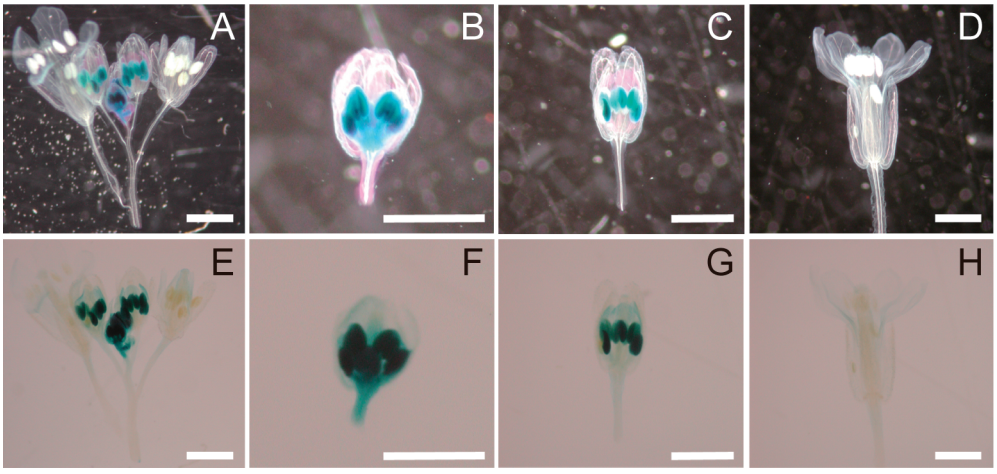

Figure 2. GUS activities of flower buds and flowers in pAS2 : GUS transgenic plants. (A–H) GUS activity in transgenic plants harbored *AS2* promoter : GUS fusion gene. (A–D) Dark-field views, (E–H) Light-field views. A, B, C and D corresponds to E, F, G and H, respectively. (A, E) inflorescence apices, (B-D, F–H) flower buds and flowers. Scale bars: 1 mm.

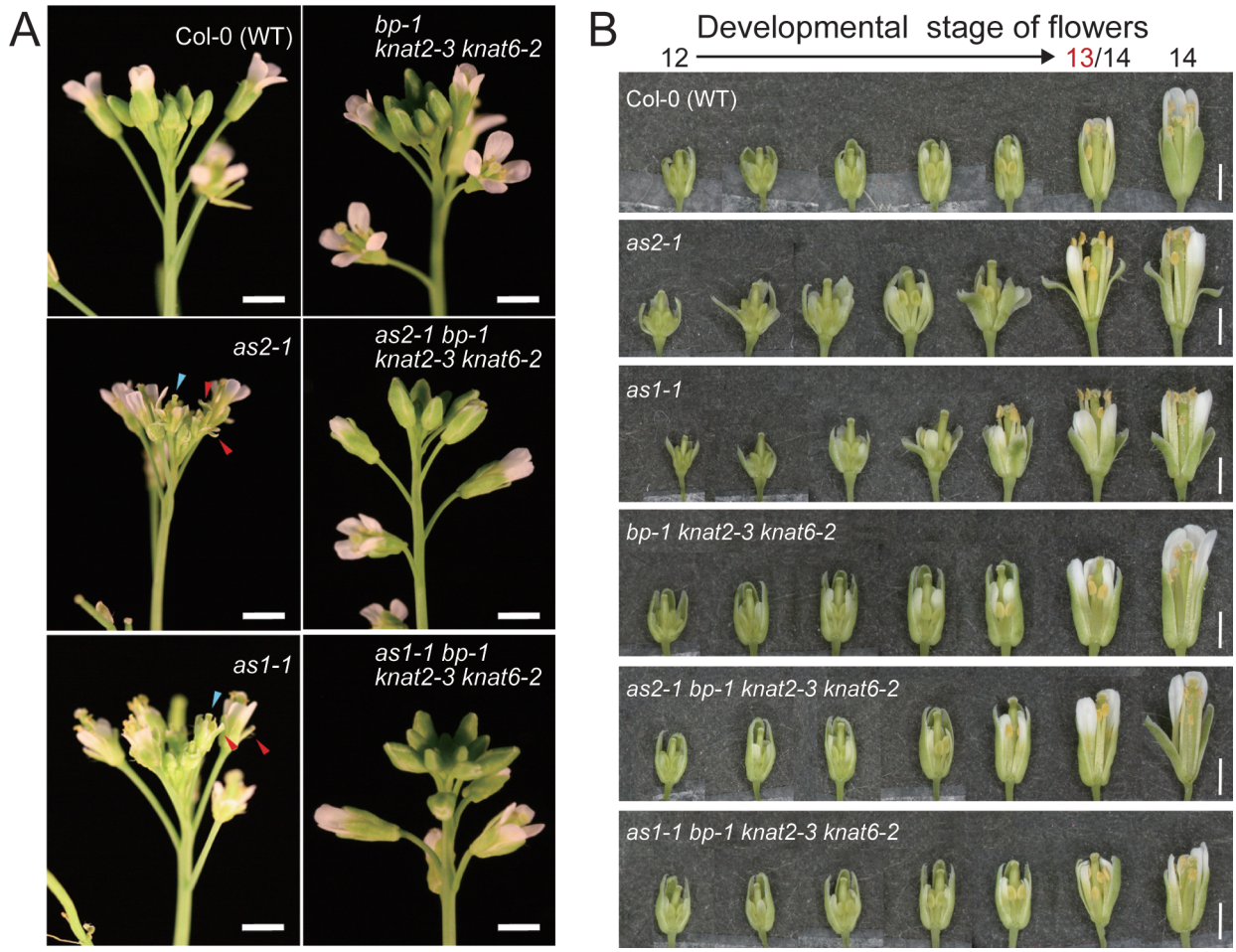

Figure 3. Morphology of floral organs in the *as2-1*, *as1-1*, *bp-1 knat2-3 knat6-2*, *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* mutants. (A) Inflorescence apices of wild type (Col-0), *as2-1*, *as1-1*, *bp-1 knat2-3 knat6-2*, *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* plants. Blue and red arrowheads indicate the stigma protrusion phenotype and the sepals that unusually be curled downwardly, respectively. (B) A series of flower buds and flowers of wild type (Col-0) and each mutant plant at developmental stages 12 to 14. The floral stage which defined as stage 13 often corresponded to the stage 14, at which the length of stamen became longer than that of carpel. Scale bars: 2 mm (A), 1 mm (B).

between the sepals continue to elongate rapidly (Smyth et al. 1990). Therefore, we tentatively fixed the stage of the flower as stage 13 when the petal length became longer than that of sepals for the first time in all mutants analyzed in this experiment. All flower buds and flowers at developmental stages 12 to 14 were collected from primary inflorescences when the first 10 flowers had opened, and the lengths of each organ were quantified (Figures 3B, 4, 5).

As shown in Figure 3, the unopened flower buds in *as2-1* and *as1-1* mutants showed the characteristic stigma-protrusion phenotype, in which stigmas were visible at the top of buds (Figure 3A, blue arrowhead). Developmental stage 12 is defined as the stage when the petal length became equal to that of the longer stamens in the wild type (Smyth et al. 1990). In late stage 12, the flower buds were fully enclosed with sepals in the wild type (Figure 3A, B). In contrast, the flower buds of *as2-1* and *as1-1* were not enclosed at the same stage (Figure 3A, B). Furthermore, the sepals were unusually curled downwardly in these mutants (Figure 3A, red arrowhead).

As shown in Figure 4A to D, we measured the lengths of the long axis of the floral organs for each flower according to the method of Tabata et al. (2010). The average lengths of sepals and petals in *as2-1* and *as1-1* mutants were shorter than those in the wild type through stages 12 to 14 (Figure 4A, B). These results confirmed the previous reports by Ikezaki et al. (2010). The average length of stamens in *as1-1* was longer than that in the wild type during the period from late stage 12 to stage 13 (Figure 4C). In *as2-1*, it showed no significant change (Figure 4C). The average carpel length in *as2-1* and *as1-1* was almost the same as that of the wild type (Figure 4D).

The balance of lengths of each floral organ was shown in Figure 5A, B. When the ratios of lengths of stamens to sepals and carpel to sepals in *as2-1* and *as1-1* were compared with those in the wild type, the ratios of lengths of carpel and stamens to sepals in *as1-1* were both higher than those in the wild type (Figure 5A, B). In

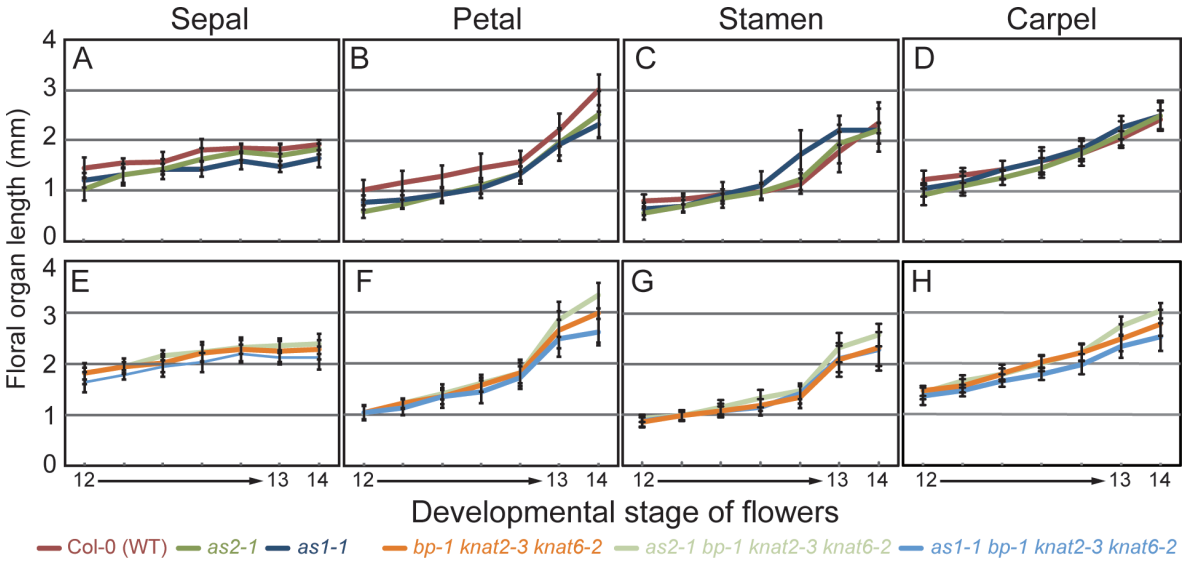

Figure 4. Floral organ length in the *as2-1*, *as1-1*, *bp-1 knat2-3 knat6-2*, *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* mutants. (A–H) The floral organ lengths of flower bud and flower that formed on the primary inflorescences in wild type, *as2-1* and *as1-1* (A–D), *bp-1 knat2-3 knat6-2*, *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* plants (E–H). The length of sepal (A, E), petal (B, F), stamen (C, G) and carpel (D, H) of each plant are represented. Numbers under the horizontal axes represent the developmental stage of each plant. Error bars indicate the standard deviations (n=11 for wild type and *as1-1* plants, n=10 for the other mutant plants).

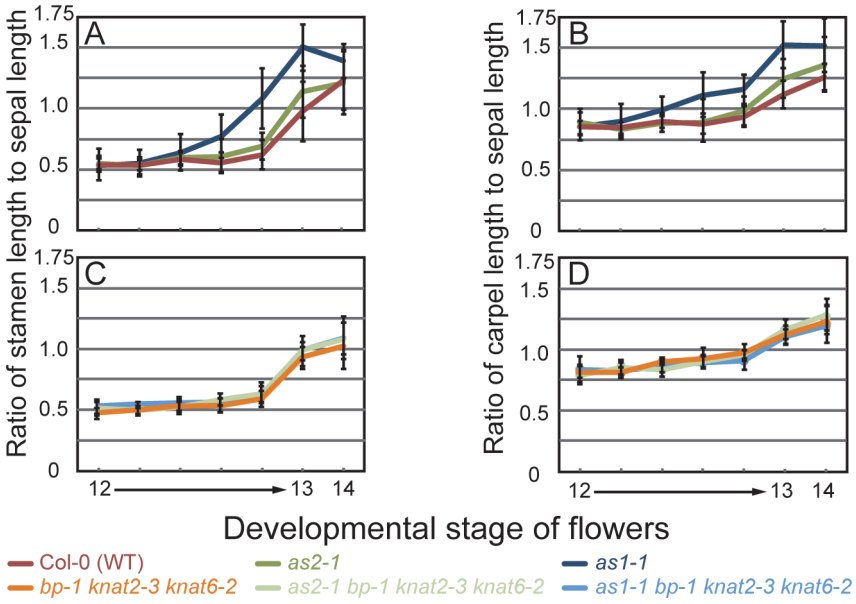

Figure 5. The ratio of the organ length against sepal length in wild type, *as2-1*, *as1-1*, *bp-1 knat2-3 knat6-2*, *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* plants. (A–D) The ratio of stamen length to sepal length (A, C) and the ratio of carpel length to sepal length (B, D) are represented. The ratio in wild type, *as2-1* and *as1-1* (A, B), *bp-1 knat2-3 knat6-2*, *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* plants (C, D). Numbers under the horizontal axes represent the developmental stage of each plant. This experiment performed one time. Error bars indicate the standard deviations (n=11 for wild type and *as1-1* plants, n=10 for the other mutant plants).

as2-1, these ratios were only a little higher than those in wild type (Figure 5A, B).

Phenotypes in as2-1 and as1-1 flowers were attributable to the ectopic expression of class 1 KNOX genes

It has been reported that the phenotype, wherein the sepals and petals of the *as2-1* and *as1-1* mutants were shorter than those of the wild type, was also suppressed by the *BP KNAT2 KNAT6* triple mutation (Ikezaki et al. 2010). To know the effect of the class 1 *KNOX* genes on the phenotypes of flower buds and flowers in the *as2* and *as1* mutation backgrounds, we quantified each floral organ length at anthesis for multiple mutants of the class 1 *KNOX* genes: *bp-1 knat2-3 knat6-2* triple mutant, *as2-1 bp-1 knat2-3 knat6-2* quadruple mutant, and *as1-1 bp-1*

knat2-3 knat6-2 quadruple mutant (Figure 4E–H).

The triple mutation of *BP KNAT2 KNAT6* genes almost suppressed the phenotypes of stigma protrusion and sepals that unusually curled downwardly in *as2-1* and *as1-1* (Figure 3A, B). The shorter lengths of sepals and petals in *as2-1* and *as1-1* were suppressed by the *BP KNAT2 KNAT6* triple mutations (Figure 4E, F). The longer length of the stamens in *as1-1* at the late stage 12 to stage 13 was also suppressed by the *BP KNAT2 KNAT6* triple mutations (Figure 4G). The ratios of lengths of stamens to sepals and carpel to sepals in *as2-1 bp-1 knat2-3 knat6-2* and *as1-1 bp-1 knat2-3 knat6-2* became the same as those in the wild type and *bp-1 knat2-3 knat6-2* (Figure 5A–D).

Discussion

In flowers, transcripts of both *AS2* and *AS1* genes were present in the same tissues at the early stages of flower development (Figure 1). From this result, *AS2* and *AS1* might act in the same process to control floral morphology. Therefore, it is supposed that the phenotype of floral organs in the *as2-1* and *as1-1* mutants might be caused by the defects in the expression of *AS2* and *AS1* in the floral meristem and organs.

Our data indicated that the change in lengths of floral organs and the ratios of lengths of stamens to sepals and carpel to sepals being larger than those in wild-type plants in stages 12 to 14 might be one explanation for the phenotype of stigma protrusion seen in *as2-1* and *as1-1* mutants (Figures 4, 5). That is, the difference from the wild type in the ratios of elongation among each floral organ in the mutants might affect the balance of the lengths of floral organs in *as2-1* and *as1-1*.

Our data showed that triple mutation of the *BP KNAT2 KNAT6* genes nearly suppressed the phenotypes of floral organs in *as2-1* and *as1-1* (Figure 3). The *as2* and *as1* mutants exhibit pleiotropic phenotypes in leaves, and some of the phenotypes of *as2-1* and *as1-1* were rescued by multiple mutations in class 1 *KNOX* genes (Ikezaki et al. 2010). It is postulated that *AS2* and *AS1* might regulate the shape of leaves through some unknown factor other than the class 1 *KNOX* genes (Ikezaki et al. 2010). In the case of reproductive organs, further analysis of the such morphological changes as the venation pattern in petals is required.

We showed that the over all phenotypes of *as2* and *as1* were similar in the floral organs. However, the phenotypes of changes in the ratios of lengths of stamens to sepals and carpel to sepals in *as2-1* were weaker than those seen in *as1-1*. There are three possible explanations for the phenotypes being weaker in *as2* than in *as1*. As the first possibility, AS2 and AS1 might independently repress the expression of class 1 *KNOX* genes. Second, *as2-1* mutant might be a weak allele. Third, other genes might be involved in the development of floral organs in addition to *AS2*. It might be possible that *AS2* and *AS1* function in floral organ development as a complex such as has been predicted in leaf development, because almost all phenotypes were similar in both *as2* and *as1* (Figure 3, our unpublished data). The phenotype of leaves in *as2-1* is similar to that in the other allele (Semiarti et al. 2001). These facts suggest that the first and second possibilities might be unlikely.

The *AS1* gene encodes a myb domain protein and is a single gene in *Arabidopsis*, whereas *AS2* belongs to the *AS2/LOB* gene family, which has 42 members in *Arabidopsis*. All members encode proteins with an AS2/LOB domain in their amino-terminal halves (Iwakawa et al. 2002; Shuai et al. 2002). Although we previously reported that *AS2* can not be functionally replaced by other members of the family during leaf development (Matsumura et al. 2009), we still do not know how *AS2* functions in floral organ development. It has been reported that *AS2* and *ASL1/LBD36*, which are members of the AS2/LOB domain gene family and the closest homologues of *AS2* (Matsumura et al. 2009), play partially redundant roles in the development of flower petals (Chalfun-Junior et al. 2005). Although no visible mutant phenotype is observed in the *asl1* loss-of-function mutant, the *asl1 as2* double mutant plants have narrower sepals resulting in exposed inner floral organs and outwards curling of sepals and petals (Chalfun-Junior et al. 2005). These phenotypes are similar to that of *as2-1* as observed in our experiment (Figure 3). Furthermore it has been shown that overexpression of *ASL1/LBD36* leads to repression of *BP* (Chalfun-Junior et al. 2005). From these data it might be possible to conclude that *AS2* and *ASL1/LBD36* have partially redundant functions in sepal and petal development. Therefore, the third possibility that other genes might be involved in the development of floral organs provides the simplest explanation.

Since the phenotype of leaves in the *as2-1 as1-1* double mutant has been shown to be similar to that of *as2-1* at the point of forming leaflet-like structures (Serrano-Cartagena et al. 1999; our unpublished results), analysis of the phenotypes of flowers in the *as2 as1* double mutant will provide some information to aid in understanding the functions of *AS2* and *AS1*.

The mutations of *ARF6* and *ARF8* concomitantly delay the elongation of floral organs and show floral phenotypes similar to those of the *as2-1* and *as1-1* mutants (Tabata et al. 2010). It has been reported that *ARF6* and *ARF8* repress the ectopic expression of class 1 *KNOX* genes in floral organs in parallel with *AS2* and *AS1*, and most floral defects in the *arf6 arf8* double mutant are attributable to the abnormal expression of class 1 *KNOX* genes (Tabata et al. 2010). Therefore, not only *AS2* and *AS1* but also *ARF6* and *ARF8* would

probably be involved in elongation of floral organs through the repression of class 1 *KNOX* genes.

Defective expression of *AS2* and *AS1* yields an altered appearance of floral morphology. *AS2* and *AS1* regulate the expression of various target genes, such as class 1 *KNOX* genes and abaxial determinant genes (Iwakawa et al. 2007). Therefore, changing the expression of *AS2* and *AS1* induces the variety of flower development regulated not only by *AS2* and *AS1* themselves but also by the downstream genes. To date, several reports have clarified the relationship between gene expression and floral phenotypes. Formation of a spur-like outgrowth, which resembled an ectopic petal tube, was dependent both on *KNOX* gene expression and dorsiventral asymmetry of the flower in *Antirrhinum* (Golz et al. 2002). The *FaKNOX1* gene from strawberry (*Fragaria* spp.) RNAi plants occasionally produced small flowers with lobed petals (Chatterjee et al. 2011). Since the AS2/LOB family is widely conserved in plants, our findings could be applicable for the molecular or biotechnological breeding of ornamental flowers.

Acknowledgements

The authors thank Sumie Ishiguro for skilled technical assistance and helpful discussions, and Minako Hirano for technical assistance. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (19060003 to Y.M.), Scientific Research (C) (21570052 to C.M., 22570053 to S.K.) from the Ministry of Education, Science, Culture and Sport of Japan; and the 'Academic Frontier' Project for Private Universities (matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology [MEXT], 2005-2009).

References

Byrne ME, Barley R, Curtis M, Arroyo JM, Dunham M, Hudson A, Martienssen RA (2000) *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* 408: 967–971

Byrne ME, Simorowski J, Martienssen RA (2002) *ASYMMETRIC LEAVES1* reveals *knox* gene redundancy in *Arabidopsis*. *Development* 129: 1957–1965

Byrne ME, Timmermans M, Kidner C, Martienssen R (2001) Development of leaf shape. *Curr Opin Plant Biol* 4: 38–43

Chalfun-Junior A, Franken J, Mes JJ, Marsch-Martinez N, Pereira A, Angenent GC (2005) *ASYMMETRIC LEAVES2-LIKE1* gene, a member of the AS2/LOB family, controls proximal-distal patterning in *Arabidopsis* petals. *Plant Mol Biol* 57: 559–575

Chatterjee M, Bermudez-Lozano CL, Clancy MA, Davis TM, Folta KM (2011) A strawberry *KNOX* gene regulates leaf, flower and meristem architecture. *PLoS ONE* 6: e24752

Golz JF, Keck EJ, Hudson A (2002) Spontaneous mutations in *KNOX* genes give rise to a novel floral structure in *Antirrhinum*. *Curr Biol* 12: 515–522

Guo M, Thomas J, Collins G, Timmermans MC (2008) Direct repression of *KNOX* loci by the ASYMMETRIC LEAVES1 complex of *Arabidopsis*. *Plant Cell* 20: 48–58

Ikezaki M, Kojima M, Sakakibara H, Kojima S, Ueno Y, Machida C, Machida Y (2010) Genetic networks regulated by *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* in leaf development in *Arabidopsis thaliana*: *KNOX* genes control five morphological events. *Plant J* 61: 70–82

Iwakawa H, Iwasaki M, Kojima S, Ueno Y, Soma T, Tanaka H, Semiarti E, Machida Y, Machida C (2007) Expression of the *ASYMMETRIC LEAVES2* gene in the adaxial domain of Arabidopsis leaves represses cell proliferation in this domain and is critical for the development of properly expanded leaves. *Plant J* 51: 173–184

Iwakawa H, Ueno Y, Semiarti E, Onouchi H, Kojima S, Tsukaya H, Hasebe M, Soma T, Ikezaki M, Machida C, Machida Y (2002) The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana*, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. *Plant Cell Physiol* 43: 467–478

Koizumi K, Sugiyama M, Fukuda H (2000) A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. *Development* 127: 3197–3204

Kojima S, Iwasaki M, Takahashi H, Imai T, Matsumura Y, Fleury D, Van Lijsebettens M, Machida Y, Machida C (2011) Asymmetric leaves2 and Elongator, a histone acetyltransferase complex, mediate the establishment of polarity in leaves of *Arabidopsis thaliana*. *Plant Cell Physiol* 52: 1259–1273

Long JA, Barton MK (1998) The development of apical embryonic pattern in *Arabidopsis*. *Development* 125: 3027–3035

Matsumura Y, Iwakawa H, Machida Y, Machida C (2009) Characterization of genes in the *ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES* (*AS2/LOB*) family in *Arabidopsis thaliana*, and functional and molecular comparisons between *AS2* and other family members. *Plant J* 58: 525–537

Ori N, Eshed Y, Chuck G, Bowman JL, Hake S (2000) Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot. *Development* 127: 5523–5532

Rédei GP, Hirono Y (1964) Linkage studies. *Arabidopsis Inf Serv* 1: 9

Semiarti E, Ueno Y, Tsukaya H, Iwakawa H, Machida C, Machida Y (2001) The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* 128: 1771–1783

Serrano-Cartagena J, Robles P, Ponce MR, Micol JL (1999) Genetic analysis of leaf form mutants from the *Arabidopsis* Information Service collection. *Mol Gen Genet* 261: 725–739

Shuai B, Reynaga-Peña CG, Springer PS (2002) The *LATERAL ORGAN BOUNDARIES* gene defines a novel, plant-specific gene family. *Plant Physiol* 129: 747–761

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in *Arabidopsis*. *Plant Cell* 2: 755–767

Tabata R, Ikezaki M, Fujibe T, Aida M, Tian CE, Ueno Y, Yamamoto KT, Machida Y, Nakamura K, Ishiguro S (2010) Arabidopsis *AUXIN RESPONSE FACTOR6* and 8 regulate jasmonic acid biosynthesis and floral organ development via repression of class 1 *KNOX* genes. *Plant Cell Physiol* 51: 164–175

Takahashi H, Iwakawa H, Nakao S, Ojio T, Morishita R, Morikawa S, Machida Y, Machida C, Kobayashi T (2008) Knowledge-based fuzzy adaptive resonance theory and the application to gene expression analysis of plants. *J Biosci Bioeng* 106: 587–593

Tanaka H, Watanabe M, Watanabe D, Tanaka T, Machida C, Machida Y (2002) *ACR4*, a putative receptor kinase gene of *Arabidopsis thaliana*, that is expressed in the outer cell layers of

embryos and plants, is involved in proper embryogenesis. *Plant Cell Physiol* 43: 419–428

Tsukaya H, Uchimiya H (1997) Genetic analyses of the formation of the serrated margin of leaf blades in Arabidopsis: combination of a mutational analysis of leaf morphogenesis with the characterization of a specific marker gene expressed in hydathodes and stipules. *Mol Gen Genet* 256: 231–238

Ueno Y, Ishikawa T, Watanabe K, Terakura S, Iwakawa H, Okada K, Machida C, Machida Y (2007) Histone deacetylases and ASYMMETRIC LEAVES2 are involved in the establishment of polarity in leaves of *Arabidopsis*. *Plant Cell* 19: 445–457