Arabidopsis AUXIN RESPONSE FACTOR6 and 8 Regulate Jasmonic Acid Biosynthesis and Floral Organ Development via Repression of Class 1 KNOX Genes

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Two mutations in Arabidopsis thaliana, auxin response factor6 (arf6) and arf8, concomitantly delayed the elongation of floral organs and subsequently delayed the opening of flower buds. This phenotype is shared with the jasmonic acid (JA)-deficient mutant dad1, and, indeed, the JA level of arf6 arf8 flower buds was decreased. Among JA biosynthetic genes, the expression level of DAD1 (DEFECTIVE IN ANOTHER DEHISCENCE1) was markedly decreased in the double mutant, suggesting that ARF6 and ARF8 are required for activation of DAD1 expression. The double mutant arf6 arf8 also showed other developmental defects in flowers, such as aberrant vascular patterning and lack of epidermal cell differentiation in petals. We found that class 1 KNOX genes were expressed ectopically in the developing floral organs of arf6 arf8, and mutations in any of the class 1 KNOX genes (knat2, knat6, bp and hemizygous stm) partially suppressed the defects in the double mutant. Furthermore, ectopic expression of the STM gene caused a phenotype similar to that of arf6 arf8, including the down-regulation of DAD1 expression. These results suggested that most defects in arf6 arf8 are attributable to abnormal expression of class 1 KNOX genes. The expression of AS1 and AS2 was not affected in arf6 arf8 flowers, and as1 and arf6 arf8 additively increased the expression of class 1 KNOX genes. We concluded that ARF6 and ARF8, in parallel with AS1 and AS2, repress the class 1 KNOX genes in developing floral organs to allow progression of the development of these organs.

Keywords: Arabidopsis thaliana • AUXIN RESPONSE FACTOR • Class 1 KNOX genes • Floral organ development • Jasmonic acid • Meristem.

Introduction

During reproductive growth in flowering plants such as Arabidopsis thaliana (Arabidopsis), a shoot apical meristem (SAM) produces floral meristems, in which floral organ primordia are formed and develop into organs by coordinated cell division and differentiation. The floral organ primordia are positioned at the auxin concentration maxima, which are established by polar localization of the auxin efflux carrier PIN-FORMED1 (PIN1) in cells (Benkova et al. 2003).
In parallel with positioning, organ primordia need to terminate stem cell maintenance and establish the identities of lateral organs. This specification process is well characterized in Arabidopsis thaliana, which is required for SAM initiation and maintenance (Long et al. 1996). The repression of STM gives rise to the expression of AS1 and AS2 in developing leaf primordia, which in turn negatively regulate the other class 1 KNOX genes, BREVIPEDICELLUS (BP), KNAT2 (for KNOTTED-like from Arabidopsis thaliana) and KNAT6 (Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001, Byrne et al. 2002, Barkoulas et al. 2007). In loss-of-function mutants of AS1 and AS2, KNOX genes are therefore ectopically expressed in leaf primordia to produce lobed leaves with abnormal vascular patterning (Byrne et al. 2000, Semiarti et al. 2001). Ectopic expression of the KNOX genes is also observed in floral organs of as1 and as2 mutants (Ori et al. 2000). Recently, it was shown that an AS1–AS2 complex binds to the BP and KNAT2 promoters and directly represses the expression of these genes (Guo et al. 2008). These findings strongly suggest that the auxin accumulated at incipient primordia must function to repress the class 1 KNOX genes. Indeed, it was reported that auxin has a role in repressing BP expression in leaves (Hay et al. 2006). However, the mechanism of repression is not yet known.

Many auxin-regulated genes have the auxin response element TGCTCTC in their promoters, to which auxin response factors (ARFs) can bind (Ulmavos et al. 1997a, Rogg and Bartel 2001, Liscum and Reed 2002, Guiffoyle and Hagen 2007). Arabidopsis has 23 genes encoding ARF proteins. Five of them, ARF5/MONOPTEROS (MP), ARF6, ARF7/NONPHOTOTROPIC HYPOCHOTYL4 (NPH4), ARF8 and ARF19, have a glutamine-rich central domain and are thought to be transcriptional activators, whereas the others are thought to be transcriptional repressors (Guiffoyle and Hagen 2007). The activity of ARFs is negatively regulated by heterodimerization with their repressors, Aux/IAA proteins, which are ubiquitinated and degraded by proteasomes in response to auxin binding to its receptor TRANSPORT INHIBITOR RESISTANT1 (TIR1) (Ulmavos et al. 1997b, Dharmasiri et al. 2005, Kepinski and Leyser 2005).

The function of each activator-type ARF has been characterized. ARF5/MP is essential for embryo axis formation and vascular development (Hardtke and Berleth 1998). ARF7/NPH4 and its most closely related ARF, ARF19, promote leaf expansion as well as lateral root formation (Okushima et al. 2005, Wilmoth et al. 2005). Double mutant plants of two paralogous factors, ARF6 and ARF8, appear normal during early vegetative growth, but, after floral induction, the leaves grow unevenly and become twisted, and the inflorescence stems elongate much less than those of the wild type. The flowers of the arf6 arf8 double mutant arrest as infertile closed buds with immature reproductive organs, indicating that ARF6 and ARF8 coordinate the transition from immature to mature fertile flowers (Nagpal et al. 2005).

Arabidopsis mutations that disrupt the genes for jasmonic acid (JA) biosynthetic enzymes such as defective in anther dehiscence1 (dadi), delayed dehiscence1 (dde1)/12-oxophytodienoate reductase3 (opr3), dde2/allene oxide synthase (aos) and triple mutations of fatty acid desaturation (fad3 fad7 fad8) as well as a JA signaling mutant, coronatine insensitive1 (coi1), show male sterility caused by defects in anther dehiscence and pollen maturation (Feyes et al. 1994, McConn and Browse 1997, Sanders et al. 2000, Stintzi and Browse 2000, Ishiguro et al. 2001, Park et al. 2002, von Malek et al. 2002). A similar phenotype was observed in plants expressing a dominant-negative form of the JASMONATE ZIM-DOMAIN1 (JAZ1) protein (Thines et al. 2007). Furthermore, Nagpal et al. (2005) reported that the arf6 arf8 double mutant has a defect in anther dehiscence, which is caused by a decrease in JA production. Thus, ARF6 and ARF8 promote the biosynthesis of JA, although the mechanism of promotion has not been identified.

In this study, we examined the function of ARF6 and ARF8 in the regulation of the JA biosynthetic pathway and found that DAD1 is a downstream target of these two ARF proteins. We also found that these proteins negatively regulate the class 1 KNOX genes in parallel with AS1 and AS2. This suppression of KNOX genes is required for development and maturation of floral organs, in part via production of JA.

### Results

**The arf6 and arf8 mutations repress floral organ elongation at flower opening**

A T-DNA-tagged Arabidopsis ARF6 gene mutant was identified from FLAGdb lines and designated arf6-101 (FLAG219_A05) (Samson et al. 2002). Although the T-DNA was inserted in the 5′-untranslated region, between the upstream open reading frames (ORFs) and the main ORF, the phenotype of arf6-101 resembled that of a reported null allele, arf6-2 (Nagpal et al. 2005), suggesting that the T-DNA insertion strongly inhibits the function of ARF6 ([Supplementary Fig. S1](#)). In comparison with the wild type, homozygotes of arf6-101 showed delayed flower opening, which resulted in the accumulation of unopened flower buds at the top of inflorescences ([Fig. 1a, b](#)) (Nagpal et al. 2005). Another T-DNA-inserted arf mutant, arf8-1, showed a phenotype similar to that of arf6-101 ([Fig. 1c](#)) (Tian et al. 2004), suggesting overlapping functions of these two genes (Nagpal et al. 2005).

The unopened flower buds of these mutants show a characteristic stigma protrusion phenotype, in which stigmas are visible at the top of buds, presumably due to the inhibited elongation of outer organs ([Fig. 1b, c](#)). To examine the effect of the
arf6 and arf8 mutations on the elongation of floral organs, all flowers and flower buds at developmental stages 12–15 (Smyth et al. 1990) were collected from primary inflorescences when the first 10 flowers had opened, and the lengths of the long axis of the floral organs of each flower were measured. In wild-type plants, initiation of rapid elongation of petals and stamens, corresponding to the beginning of stage 13, was observed at the seventh flower (numbered from the youngest stage 12 flower) (Fig. 1g). In arf6-101 and arf8-1 mutants, the beginning of rapid organ growth was delayed by 2–3 flowers. During this delay, the gradual elongation of carpels caused them to extend past the other three organs, in turn causing the stigma protrusion phenotype (Fig. 1g).

Compared with each single mutant, the petals and stamens of the arf6-101 arf8-1 double mutant lacked this rapid growth, resulting in a defect in flower opening (Fig. 1d) (Nagpal et al. 2005). Gradual elongation of carpels led to stigma-protruding flower buds; this phenotype remained until senescence (Fig. 1d, g). In initially produced flowers the carpels of the double mutants were shorter than those of the wild type (Fig. 1d, g), but the expansion of pistils became prominent in the flowers that were produced at a later stage (Fig. 2a, b).
arf6 arf8 double mutants repress the DAD1 gene expression required for JA production and floral organ elongation

The JA biosynthesis mutant, dad1-1, as well as other JA-deficient and JA-insensitive mutants, makes a certain number of stigmatic protruding flower buds (Fig. 1e, f), and frequently shows developmental arrest of flower opening, a phenotype resembling that of the arf6-101 arf8-1 double mutant (Supplementary Fig. S2). We measured the floral organ lengths of dad1-1 at anthesis, revealing a delay of floral organ elongation similar to that observed in arf6-101 and arf8-1 mutants (Fig. 1h).

The level of JA in the arf6-101 arf8-1 flower bud clusters was one-third that of the wild type (Fig. 2d), as reported (Nagpal et al. 2005), suggesting the involvement of JA deficiency in floral organ elongation of the double mutant. When we applied exogenous JA to the arf6-101 arf8-1 flower buds, partial rescue of petal elongation was observed (Fig. 2b, c). Furthermore, a failure of anther dehiscence at flower opening observed in the double mutants, which is another typical phenotype of JA-deficient mutants, was also restored by JA treatment (data not shown).

To look for the JA biosynthetic genes regulated by ARF6 and ARF8, we prepared total RNA from flower buds and carried out quantitative reverse transcription–PCR (RT-PCR). In this experiment, flower bud clusters at inflorescence apices including apical meristems, all flower buds and the youngest opened flowers were used for the wild type, whereas inflorescence apices above extended internodes (including apical meristems, flower buds and some unopened flowers) were used for the double mutant. Among the five genes (DAD1, LOX2, AOS, AOC4 and OPR3) encoding the enzymes of the JA biosynthetic pathway, only the level of DAD1 transcript was dramatically decreased in the flower buds of arf6-101 arf8-1 double mutants (Fig. 2e–i). To confirm the down-regulation of DAD1 expression by using a β-glucuronidase (GUS) reporter gene, a DAD1 promoter–GUS construct (P_DAD1-GUS) was introduced into plants and GUS expression was observed. In contrast to the intense expression of P_DAD1-GUS in the filaments of wild-type flowers (Fig. 2j) (Ishiguro et al. 2001, Nakagawa et al. 2007), all arf6-101 arf8-1 plants carrying the transgene (n = 12) showed only restricted GUS staining at the base of filaments (Fig. 2k). Thus, it appears that ARF6 and ARF8 are required for the activation of DAD1 expression in developing flower buds. We then introduced the DAD1 coding sequence attached to the PISTILLATA promoter (P_PI), the activity of which continues during stamen and petal development (Honma and Goto 2000), into the arf6-101 arf8-1 double mutant. Although no obvious restoration of the phenotype was observed, the level of JA in the flower buds partly recovered (Fig. 2d). This result suggests that the decreased expression of DAD1 in the arf6-101 arf8-1 double mutant accounts at least in part for the reduction in JA level in the flower buds.

ARF6 and ARF8 are required for the differentiation of floral organs

The elimination of DAD1 expression in arf6-101 arf8-1 double mutants may have reflected an earlier defect in floral organ development. Thus we examined the morphology of petals. Microscopic observation revealed that the petals of arf6-101 arf8-1 double mutants showed abnormal patterns of vascular bundles. Wild-type petals contained a central primary vein, which extended along the long axis of the petal, and 2–4 secondary veins, which formed the vascular loops (Fig. 3a). Petals of arf6-101 arf8-1 double mutants contained an intact
primary vein, but secondary veins rarely formed a vascular loop (Fig. 3b). Because inhibitors of polar auxin transport affect vascular patterning in leaves (Mattsson et al. 1999, Sieburth 1999), we treated wild-type flower buds with one such inhibitor, N-1-naphthylphthalamic acid (NPA). The petals that formed on NPA-treated buds showed an aberrant pattern of secondary veins that was quite similar to that of arf6-101 arf8-1 petals, suggesting that the function of ARF6 and ARF8 in vascular patterning may be attributable to auxin signaling (Fig. 3c, d).

The adaxial surface of fully developed wild-type petal blades (i.e. the distal half of the petals) consists of characteristic conically shaped epidermal cells, whereas the abaxial surface, as well as both surfaces of young developing petals, is filled with relatively flat cells (Fig. 3e, g). However, both surfaces of arf6-101 arf8-1 double mutant petals consisted of flat cells (Fig. 3f, h), suggesting that the differentiation of epidermal cells does not proceed to a sufficient extent in the mutant. Alternatively, it is possible that the ARF6 and ARF8 genes are involved in adaxial/abaxial specification; however, the abaxial-specific expression of a FILAMENTOUS FLOWER promoter (P_FIL)-GFP (green fluorescent protein) construct (Watanabe and Okada 2003) was not affected in the double-mutant petals (data not shown). It should be noted that the abnormalities of vascular patterning and epidermal cell differentiation, as well as pistil expansion, were not restored by JA treatment, suggesting that the JA deficiency is not always responsible for the morphological abnormalities in the double mutants.

**ARF6 and ARF8 negatively regulate class 1 KNOX genes in flowers**

Ecctic expression of class 1 KNOX genes strongly inhibits the elongation and differentiation of floral organs (Ori et al. 2000, Pautot et al. 2001, Borghi et al. 2007, Scofield et al. 2007). Because these characteristics were shared with those of arf6-101 arf8-1 double mutants, we quantified the expression levels of class 1 KNOX genes. Quantitative RT–PCR revealed that the transcript levels of all class 1 KNOX genes, STM, BP, KNAT2 and KNAT6, were increased in arf6-101 arf8-1 flower buds (Fig. 4a–d). The GUS gene driven by the STM promoter (P_STM–GUS) in wild-type plants was strongly expressed in the SAM (Fig. 4e), which is consistent with the reported localization of STM mRNA (Long et al. 1996). Weak expression was also observed in the developing carpels and at the base of stamens and carpels (Fig. 4e, g). In contrast, strong P_STM–GUS expression in arf6-101 arf8-1 double mutants extended into all organs of developing flower buds (Fig. 4f, h). These results indicated that ARF6 and ARF8 are required to repress the expression of class 1 KNOX genes in developing floral organs. It is consistent with the expression of ARF6 and ARF8 genes in all floral organs (Nagpal et al. 2005). In contrast, the expression levels of these KNOX genes in leaves did not differ between the wild type and arf6-101 arf8-1 mutant, suggesting that a different regulatory mechanism exists in leaves (Supplementary Fig. S3).

**Phenotypes in arf6 arf8 flowers are attributable to the ectopic expression of class 1 KNOX genes**

To examine the genetic interactions among ARF6, ARF8 and class 1 KNOX genes, we attempted to make triple mutants. Each single mutant of knat2-3, knat6-2 and bp-1 makes flowers indistinguishable from wild-type flowers, except that bp-1 flowers point downward. The flowers of arf6-101 arf8-1 knat2-3 and arf6-101 arf8-1 knat6-2 triple mutants never opened, like those of the arf6-101 arf8-1 double mutant, but the stigma-protruding phenotype was suppressed (Fig. 5a–d). This suppression was mainly due to the reduced pistil expansion. The flowers of arf6-101 arf8-1 bp-1 looked similar to those of arf6-101 arf8-1, although the pistils were slimmer (Fig. 5e–h). A triple mutant of arf6-101, arf8-1 and bumbershoot1-3 (bum1-3), which is a weak allele of stm, was lethal at the seedling stage. Instead, the semi-triple mutant (double mutant containing a hemizygous third mutation) arf6-101 arf8-1

Fig. 3 Developmental defects of arf6-101 arf8-1 petals. (a–d) Venation patterns of fully expanded petals of (a, c, d) the wild type and (b) arf6-101 arf8-1. (c) and (d) represent the petals after mock and NPA treatments, respectively. The arrow and arrowheads indicate a primary vein and vascular loops, respectively. Bars, 0.5 mm. (e, f) Scanning electron micrographs of the adaxial surface of fully expanded petals of (e) wild type and (f) arf6-101 arf8-1. Bars, 50 μm. (g, h) Transverse sections of (g) wild-type and (h) arf6-101 arf8-1 petals. ad, adaxial surface; ab, abaxial surface. Bars, 150 μm.
STM/bum1-3 showed a much less extensive pistil expansion phenotype than other triple mutants (Fig. 5i), although the single hemizygous mutation STM/bum1-3 had no effect on flower morphology. Even though the length of petals and stamens was not restored in unopened flowers of the semi-triple mutant, anther dehiscence was frequently observed at the anthesis stage (Fig. 5j–l). Moreover, DAD1 expression was increased in arf6-101 arf8-1 STM/bum1-3 flower buds relative to the arf6-101 arf8-1 double mutant (Fig. 5m). These observations indicated that abnormalities in the arf6-101 arf8-1 double mutant are at least partly suppressed by an additional mutation in class 1 KNOX genes.

Conversely, another semi-triple mutant, arf6-101 ARF8/arf8-1 bum1-3, formed inflorescences with many flowers, whereas each inflorescence of the bum1-3 single mutant produced few flowers and set a terminal flower (Fig. 5n–p). This result indicated that the arf6 and arf8 mutations suppress the stm mutation in the inflorescence meristem, probably due to enhanced expression of the partially functional bum1-3 allele or up-regulation of other class 1 KNOX genes. However, the flowers of this semi-triple mutant, as well as bum1-3 flowers, lacked pistils and never set seed. This suppression is similar to the finding that as1 can partially rescue stm in embryonic and vegetative meristems (Byrne et al. 2000).

We then examined whether ectopic expression of STM in floral organs mimics the phenotype of arf6-101 arf8-1 double mutants. Because the constitutive expression of STM is lethal at the young seedling stage, we used plants in which STM activity can be induced in flower buds. These plants have a gene encoding a fusion protein of STM and the hormone-binding domain of the rat glucocorticoid receptor (STM-GR) driven under the control of a constitutively active cauliflower mosaic virus (CaMV) 35S promoter (Brand et al. 2002). In the absence of

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**Fig. 4** Ectopic expression of class 1 KNOX genes in arf6 arf8 flowers. (a–d) Quantitative RT–PCR analysis of (a) STM, (b) BP, (c) KNAT2 and (d) KNAT6 expression in flower buds of the wild type (white) and arf6-101 arf8-1 (gray). Error bars indicate the standard deviations (n = 3). (e–h) Expression of PSTM-GUS in (e) wild-type and (f) arf6-101 arf8-1 inflower apices, (g) a wild-type stage-12 flower bud and (h) an unopened arf6-101 arf8-1 flower. Bars, 1 mm.

**Fig. 5** Genetic interactions among ARF6, ARF8 and class 1 KNOX genes. (a–e) Inflorescence apices of (a) the wild type, (b) arf6-101 arf8-1, (c) arf6-101 arf8-1 knat2-3, (d) arf6-101 arf8-1 knat6-2 and (e) arf6-101 arf8-1 bp-1. (f–i) Fully opened (stage 14) or equivalently grown flowers of (f) the wild type, (g) arf6-101 arf8-1, (h) arf6-101 arf8-1 bp-1 and (i) arf6-101 arf8-1 STM/bum1-3. (j–l) Anthers of fully developed (j) wild-type, (k) arf6-101 arf8-1 and (l) arf6-101 arf8-1 STM/bum1-3 flowers. (m) Quantitative RT–PCR analysis of DAD1 expression in flower buds of arf6-101 arf8-1 (gray) and arf6-101 arf8-1 STM/bum1-3 (black). Results indicate levels relative to those obtained for arf6-101 arf8-1 plants. Error bars indicate the standard deviations (n = 3). (n–p) Inflorescence apices of (n) the wild type, (o) bum1-3 and (p) arf6-101 ARF8/arf8-1 bum1-3. (o) indicates a terminal flower. Bars, 1 mm in (a–i) and (n–p), and 0.2 mm in (j–l).
dexamethasone (DEX) treatment, the plants produced normal flowers (Fig. 6a). When we treated their flower bud clusters with DEX to activate STM-GR, petals and stamens were shortened and sepals were almost absent (Fig. 6b), as reported previously (Borghi et al. 2007). We found that incomplete vascular loops of petals and defects in anther dehiscence, which are characteristic of arf6 arf8 flowers, were frequently observed after DEX treatment (Fig. 6b–d). The level of DAD1 expression was reduced in the DEX-treated flower buds, whereas no difference was observed in the expression of other JA biosynthetic genes (Fig. 6e). Similar morphological abnormalities, though to differing extents, were observed in flowers ectopically expressing other class 1 KNOX genes under the control of the CaMV 35S promoter (Supplementary Fig. S4). The flowers of P35S-KNAT6 plants most resembled arf6-101 arf8-1 flowers. The amount of DAD1 mRNA was also decreased in flower buds of these plants (Supplementary Fig. S4). These observations indicated that many aspects of the arf6-101 arf8-1 phenotype in flowers are attributable to the ectopic expression of class 1 KNOX genes.

ARF6 and ARF8 function in parallel with AS1 to regulate flower development

AS1 and AS2 repress the expression of class 1 KNOX genes in developing floral organs, as well as in developing leaves (Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001, Byrne et al. 2002). The floral organs of the as1 mutant are small and have abnormal vascular patterns (Semiarti et al. 2001), resembling the characteristics of the arf6-101 arf8-1 double mutant. To investigate the genetic interaction among ARF6, ARF8 and AS1, we constructed an arf6-101 arf8-1 as1-1 triple mutant. The triple mutant showed a strong dwarf phenotype (Fig. 7a). Its floral organs were smaller than those of either the as1-1 mutant or the arf6-101 arf8-1 double mutant (Fig. 7b–f). The vascular arrangement in petals was also severely affected in the arf6-101 arf8-1 as1-1 triple mutant (Fig. 7g). The transcript levels of all four class 1 KNOX genes were increased additively by a combination of the arf6-101 arf8-1 and as1-1 mutations, indicating an obvious relationship between the extent of floral organ abnormality and the levels of KNOX gene expression (Fig. 7h). In addition, the expression levels of AS1 and AS2 genes were not affected by the arf6-101 arf8-1 double mutation, indicating that the defects of the arf6-101 arf8-1 double mutant are not a consequence of the down-regulation of AS1 or AS2 (Fig. 7i). Conversely, the transcript levels of ARF6 and ARF8 were not affected by the as1-1 mutation (Fig. 7j). We concluded that ARF6 ARF8 and AS1 AS2 function in parallel to repress the expression of class 1 KNOX genes, which results in the proper development of floral organs.

Discussion

ARF6 and ARF8 are required for the activation of JA biosynthesis

The petals and stamens of arf6-101 and arf8-1 show delay of elongation at anthesis, which results in the stigma protrusion phenotype of unopened flower buds. This phenotype is much enhanced in arf6-101 arf8-1 double mutants and closely resembles that of JA-deficient and JA-insensitive mutants. A defect in anther dehiscence, which is another typical phenotype in JA-defective mutants, also appeared in arf6-101 arf8-1 double mutants. In fact, the endogenous JA level in the flower buds of the double mutants was lower than in the wild type. Among the genes encoding the enzymes of the JA biosynthetic pathway, only the expression of DAD1 decreased in the double mutant flower buds, and the JA level in arf6-101 arf8-1 flowers was partially restored by the expression of DAD1 under the P35S promoter. Therefore, we concluded that ARF6 and ARF8 are essential for the activation of DAD1 expression in developing flower buds, and consequently for the production of JA that is required for flower opening and anther dehiscence. Nagpal et al. (2005), however, reported that several JA biosynthetic pathway genes, including LOX2, AOS and OPR3, are underexpressed 2- to 3-fold in arf6 arf8 flower buds, whereas no alteration in the level of DAD1 expression was observed. We prepared

Fig. 6 Effects of ectopic expression of STM gene on floral organ development. (a, b) Fully opened flowers detached from P35S-STM-GR inflorescences 7 d after treatment with (a) mock solvent and (b) DEX. Bars, 0.5 mm. (c, d) Venation patterns of fully expanded petals of P35S-STM-GR plants 7 d after treatment with (c) mock solvent and (d) DEX. Bars, 0.5 mm. (e) Quantitative RT-PCR analysis of genes for JA biosynthetic enzymes in flower buds of P35S-STM-GR plants 3–5 d after treatment with (white) mock solvent and (gray) DEX. Error bars indicate the standard deviations (n = 3).

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The petals and stamens of arf6-101 and arf8-1 show delay of elongation at anthesis, which results in the stigma protrusion phenotype of unopened flower buds. This phenotype is much enhanced in arf6-101 arf8-1 double mutants and closely resembles that of JA-deficient and JA-insensitive mutants. A defect in anther dehiscence, which is another typical phenotype in JA-defective mutants, also appeared in arf6-101 arf8-1 double mutants. In fact, the endogenous JA level in the flower buds of the double mutants was lower than in the wild type. Among the genes encoding the enzymes of the JA biosynthetic pathway, only the expression of DAD1 decreased in the double mutant flower buds, and the JA level in arf6-101 arf8-1 flowers was partially restored by the expression of DAD1 under the P35S promoter. Therefore, we concluded that ARF6 and ARF8 are essential for the activation of DAD1 expression in developing flower buds, and consequently for the production of JA that is required for flower opening and anther dehiscence. Nagpal et al. (2005), however, reported that several JA biosynthetic pathway genes, including LOX2, AOS and OPR3, are underexpressed 2- to 3-fold in arf6 arf8 flower buds, whereas no alteration in the level of DAD1 expression was observed. We prepared
ARF6 and ARF8 repress class 1 KNOX genes in floral organs in parallel with AS1 and AS2

Meristem maintenance and lateral organ specification are regulated by antagonistic activities between AS1 AS2 and class 1 KNOX genes (Barkoulas et al. 2007). Therefore, the class 1 KNOX genes are ectopically expressed in lateral organs of as1 and as2, which accounts for the morphological abnormalities in leaves and floral organs of these mutants. We found that the phenotypes of arf6-101 arf8-1 flowers, including the JA-deficient phenotype described above, also resemble those of as1 and as2, and that all four class 1 KNOX genes are up-regulated in arf6-101 arf8-1 flower buds. Moreover, the absence of DAD1 expression in the double mutants, which can be partly rescued by a mutation in any class 1 KNOX gene, was mimicked in wild-type flower buds when STM expression was ectopically induced. These observations indicate that, like AS1 and AS2, ARF6 and ARF8 repress class 1 KNOX genes in floral organs. This finding is consistent with the expression of ARF6 and ARF8 genes in all floral organs including stamen filaments (Nagpal et al. 2005), where PSTM-GUS is expressed and PSTM-GUS is suppressed. It is notable that the expression levels of AS1 and AS2 were not altered in arf6-101 arf8-1, and that the as1 mutation did not affect the mRNA levels of ARF6 and ARF8. Moreover, as1 and arf6-101 arf8-1 showed additive effects on the ectopic expression of class 1 KNOX genes. Taken together, we conclude that ‘ARF6 and ARF8’ and ‘AS1 and AS2’ comprise a set of parallel pathways that negatively regulate class 1 KNOX genes in developing floral organs.

Based on structural similarities to ARF5/MP and ARF7/NPH4, which are transcriptional activators interacting with the canonical auxin response element, ARF6 and ARF8 are predicted to have a similar function (Guilfoyle and Hagen 2007, Okushima et al. 2007, Cole et al. 2009). To date, there is no evidence that these types of ARFs are directly involved in transcriptional repression. Therefore, down-regulation of class 1 KNOX genes might be an indirect effect of ARF6 and ARF8, although canonical auxin response elements are located upstream of STM and other class 1 KNOX genes.

RNA for expression analysis from opening flower buds (stage 13) and just-opened flowers (stage 14), in which JA biosynthesis is maximally activated (Ishiguro et al. 2001), whereas only younger (stages 11 and 12) flower buds were used in the earlier study. Furthermore, Nagpal et al. (2005) used microarrays to analyze expression, but we have found that the DAD1 signal in Affymetrix microarrays often does not reflect the true levels of its expression. These differences in starting material and technique might explain the differences between the two reports.

An involvement of JA deficiency in the phenotype of arf6 arf8 flowers was also shown by our JA feeding experiment, in which exogenously applied JA caused petals to elongate and anthers to dehisce. However, JA treatment never rescued several other phenotypes, such as pistil expansion, vascular patterning in petals and epidermal cell shape in petals. These observations are consistent with JA-deficient and JA-insensitive mutants never showing such phenotypes. Therefore, we conclude that the activation of JA biosynthesis is only part of the function of ARF6 and ARF8 in developing flower buds.
In spite of the extensive morphological defects in flowers, the alteration of leaf morphology was less severe in \textit{arf6-101 arf8-1}. This observation indicates that the function of ARF6 and ARF8 is predominant in floral organs, which is consistent with the flower-predominant expression of these genes (Nagpal et al. 2005). It has been reported that ARF7/NPH4 and ARF19, which also shows high structural similarity to ARF6 and ARF8, promote leaf expansion as well as lateral root formation (Okushima et al. 2005, Wilmoth et al. 2005). ARF7 and ARF19 are expressed throughout vegetative and reproductive organs, including leaves and roots, suggesting that ARF6 and ARF8 are used in floral organs while ARF7 and ARF19 are used in vegetative organs.

BP, KNAT2 and KNAT6 are ectopically expressed in leaves of \textit{as1} and \textit{as2}, whereas no expression of STM is observed (Byrne et al. 2000, Ori et al. 2000, Byrne et al. 2002). Thus, STM is thought to function differently from other class 1 KNOX genes. It has been proposed that STM is positioned upstream of \textit{AS1} and \textit{AS2} and is involved in meristem maintenance, whereas other class 1 KNOX genes work downstream of \textit{AS1} and \textit{AS2} (Byrne et al. 2002). Recently, it was reported that the \textit{AS1}–\textit{AS2} complex directly binds to the promoters for BP and KNAT2 to silence these genes, whereas the STM promoter lacks this binding motif (Guo et al. 2008). Moreover, STM is required to promote or maintain BP and KNAT2 expression in floral meristems (Sc澧field et al. 2007). However, our observations suggest that the expression of all four class 1 KNOX genes, including STM, is down-regulated similarly in floral organs by an additive function of \textit{AS1} \textit{AS2} and ARF6 ARF8. It seems likely that the hierarchical difference among the class 1 KNOX genes observed in leaves disappears in floral organs, where rather they are regulated equally.

**Down-regulation of class 1 KNOX genes is responsible for the development of floral organs**

As mentioned above, ARF6 and ARF8 are required for the transcriptional activation of \textit{DAD1} in developing floral organs and subsequently for JA biosynthesis and flower opening. It seems likely that \textit{DAD1} is not a direct target of ARF6 and ARF8, but rather is regulated by way of suppression of class 1 KNOX genes, because the defect in \textit{DAD1} expression was also observed in flower buds in which a class 1 KNOX gene was ectopically activated.

The morphology of flowers that ectopically express each of the four class 1 KNOX genes obviously differed, suggesting that each class 1 KNOX gene has specific functions for the development of different floral organs. All of the class 1 KNOX genes were strongly expressed in \textit{arf6-101 arf8-1} double and \textit{arf6-101 arf8-1 as1-1} triple mutant flowers, the phenotype of which appears to be due to an additive effect of the ectopic expression of all class 1 KNOX genes.

In developing leaves, suppression of class 1 KNOX genes is required for the transcriptional activation of the gibberellin biosynthesis gene \textit{AtGA20ox1} (Hay et al. 2002). Exogenously supplied gibberellin suppresses the misexpression phenotype of the \textit{BP} gene in Arabidopsis leaves (Hay et al. 2002). However, the level of \textit{AtGA20ox1} mRNA was only slightly decreased in \textit{arf6 arf8} flower buds compared with the wild type, suggesting that class 1 KNOX genes are not involved in gibberellin biosynthesis in floral organs (data not shown).

The vascular patterning in leaves is controlled by polar auxin transport, which depends on the subcellular localization of PIN1 protein (Scarpella et al. 2006). Conversely, the expression of the PIN1 gene is regulated by the level of auxin (Scarpella et al. 2006). The petals of \textit{arf6-101 arf8-1} double mutants show incomplete vascular patterning, which is quite similar to the appearance of wild-type petals treated with NPA, an inhibitor of auxin transport. A similar phenotype is observed in the petals of plants that ectopically express the class 1 KNOX genes. These observations suggest that the formation of vascular patterning, and probably the appropriate expression of a \textit{PIN1} gene, are dependent on the suppression of class 1 KNOX genes, which might be under the regulation of auxin signaling transmitted by ARF6 and ARF8.

Initials of lateral organs are defined by local auxin concentration maxima generated by activity of the \textit{PIN1} protein at the SAM (Benkova et al. 2003, Reinhardt et al. 2003, Heisler et al. 2005). Live imaging analysis of Arabidopsis inflorescence SAM revealed that \textit{STM} expression is down-regulated at locations where \textit{PIN1} expression is up-regulated (Heisler et al. 2005). However, the mechanism of how the accumulated auxin down-regulates the STM has not been elucidated. We found that the inflorescence SAM activity in a weak \textit{stm} (\textit{bum1-3}) mutant was partly recovered in the presence of the \textit{arf6-101 ARF8/arf8-1} mutations, suggesting that ARF6 and ARF8 are also involved in the regulation of SAM activity. This observation may be a clue to understand the function of auxin in the SAM.

**Materials and Methods**

**Mutants and background strains**

\textit{arf6-101} (FLAG219\_A05) was identified from the \textit{FLAGdb/FST} collection (Samson et al. 2002), \textit{arf8-1}, \textit{bp-1} and \textit{dad1-1} were previously described (Ishiguro et al. 2001, Douglas et al. 2002, Tian et al. 2004). \textit{as1-1} (CS3374), \textit{bum1-3} (CS3781), \textit{knat2-3} (SALK\_004073) and \textit{knat6-2} (SALK\_054482) were obtained from the Arabidopsis Biological Resource Center (\url{http://www.arabidopsis.org/}). \textit{arf6-101, arf8-1} and \textit{dad1-1} were derived from the Wassilewskija (Ws) strain. \textit{as1-1}, \textit{bum1-3}, \textit{knat2-3} and \textit{knat6-2} were from the Columbia (Col-0) strain. \textit{bp-1} derived from the Landsberg erecta strain was outcrossed with Col-0 three times before use in experiments. Plants were grown under continuous white light at 20–22°C.

**Transgenic plants**

Plants harboring the \textit{P\textsubscript{PI}/DAD1:GFP} gene were created as follows. The \textit{PI} promoter and \textit{DAD1} coding sequence (cds) were PCR amplified from wild-type (Col-0) genomic DNA using the primers listed in \textit{Supplementary Table S1}. The amplified \textit{PI}

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promoter and DAD1 cds were cloned into pDONR P4-P1R and pDONR201, respectively, using Gateway BP clonase (Invitrogen Corp., http://www.invitrogen.com). The inserts of the two entry clones were then integrated into the binary vector R4pGWB450 (Nakagawa et al. 2008) by LR clonase (Invitrogen), and the resultant plasmid was used for Agrobacterium-mediated transformation of arf6-101 ARF8/arf8-1 plants. Transformants were selected by kanamycin, and arf6-101 arf8-1 double mutants were selected by genotyping. To construct PSTM>GUS, a 4.4 kb STM sequence immediately upstream of the start codon, a GUS gene derived from pBII101, and a 0.8 kb STM sequence immediately downstream of the stop codon were amplified by PCR (Supplementary Table S1) and combined into pBIN50, a modified vector derived from pBIN19 (Frisch et al. 1995). The construct was transformed into wild-type Arabidopsis (Ws), and transgenic plants were selected by kanamycin. For the PSTM>GUS, PSTM(−2901+65)GUS (Col-0) was used (Nakagawa et al. 2007). arf6-101 arf8-1 double mutants carrying the PSTM>GUS (or PSTM,GUS) transgene were identified in T2 seedlings derived from a cross between arf6-101 ARF8/arf8-1 and wild-type plants containing PSTM>GUS (or PSTM,GUS). PSTM,STM-GR plants (Col-0) were kindly provided by Dr. Rüdiger Simon (Brand et al. 2002). PSTM,BP, PSTM,KNAT2 and PSTM,KNAT6 plants (Col-0) were described previously (Ikezaki et al. 2009). Detailed information about the construct will be provided upon request.

**Treatment with chemicals**

For treatment with an auxin transport inhibitor, inflorescence apices were dipped twice in a solution of 100 µM NPA (Tokyo Chemical Industry Co. Ltd, http://www.tcieurope.eu/en/) containing 0.01% of the surfactant Silwet L-77 (Momentive Performance Materials Inc., http://www.momentive.com) with an 8–10 h interval. Mock treatments were performed with distilled water containing 0.01% Silwet L-77. Flowers were observed 10–14 d after treatment. To apply JA, inflorescence apices were dipped in a solution of 0.5 mM methyl jasmonate (Wako Pure Chemical Industries, http://www.wako-chem.co.jp/english/) containing 0.05% Tween-20.

For DEX treatment of STM-GR plants, inflorescence apices were dipped twice in a solution of 20 µM DEX (Wako Pure Chemical Industries) containing 0.01% Silwet L-77 with an 8–10 h interval. Mock treatments were performed with distilled water containing 0.01% Silwet L-77. Flower buds together with the youngest opened flowers were harvested 3–5 d after treatment for RNA preparation.

**Observation of plant morphology and gene expression**

An SXZ12 microscope and BX60 microscope (Olympus Corp., http://www.olympus.co.jp/en/) were used. To observe vascular patterning, petals were cleared in 8:1:2 (w/v/v) chloral hydrate : glycerol : water. Cross-sections of petals embedded in Technovit resin (Heraeus Kulzer GmbH http://www.kulzer-technik.de) were prepared as described in Inagaki et al. (2006).

To observe petal epidermal cells, detached organs were directly observed with an S-3000N scanning electron microscope equipped with a cool stage (Hitachi High-Technologies Corp., http://www.hitachi-hitec.com/global/). GUS staining was performed as described in Ishiguro et al. (2001).

**Measurement of mRNA**

RNA was extracted from 50–100 mg of tissue using an RNeasy Plant Mini Kit and RNase-free DNase Set (QIAGEN, http://www.qiagen.com), and then used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primers. Quantitative PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, http://www.appliedbiosystems.com) with the Power SYBR Green PCR Master Mix (Applied Biosystems). The primers used for PCR are indicated in Supplementary Table S1. Levels of each mRNA were normalized to the levels of PDF2 (Atg13320) mRNA (Czechowski et al. 2005), with the value from wild-type plants (or mock-treated plants) arbitrarily set to 1.

**Measurement of JA**

Approximately 200 mg of flower bud clusters (including apical meristem, all flower buds and the youngest opened flower) was weighed, frozen in liquid nitrogen, ground with a mortar and pestle, and extracted with 4 ml of 70% (v/v) aqueous methanol. For arf6-101 arf8-1 mutants, inflorescence apices above extended internodes were considered as an equivalent to the flower bud clusters of the wild type. As an internal standard, 0.1 nmol of (+/-)-jasmonic acid-9,10-[2 H] (Tokyo Chemical Industry) was added. The extract was concentrated by vacuum concentrator and 1 µl of formic acid (FA) was added to it. After removing any debris by centrifugation, the supernatant was applied onto a Sep-Pak C18 cartridge (Waters Corp, http://www.waters.com). The cartridge was washed with 3 ml of 0.1% FA and 3 ml of 30% acetonitrile (ACN)/0.1% FA. Then the JA fraction was eluted with 3 ml of 50% ACN/0.1% FA and concentrated to 600 µl by a vacuum concentrator.

Liquid chromatography was performed with a model PU-2085 HPLC system (JASCO International Co., Ltd., http://www.jasco.co.jp) operated in negative-ion electrospray ionization ion-trap mass spectrometer (Thermo Scientific, http://www.thermo.com) operated in negative-ion mode, and detected as a combination of m/z values 209/59 (Kiribuchi et al. 2005).

**Supplementary data**

Supplementary data are available at PCP online.
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**References**


