

# Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation

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## Summary

Pollination in flowering plants requires that anthers release pollen when the gynoecium is competent to support fertilization. We show that in *Arabidopsis thaliana*, two paralogous auxin response transcription factors, ARF6 and ARF8, regulate both stamen and gynoecium maturation. *arf6 arf8* double-null mutant flowers arrested as infertile closed buds with short petals, short stamen filaments, undehisced anthers that did not release pollen and immature gynoecia. Numerous developmentally regulated genes failed to be induced. ARF6 and ARF8 thus coordinate the transition from immature to mature fertile flowers. Jasmonic acid (JA) measurements and JA feeding experiments showed that decreased jasmonate production caused the block in pollen release, but not the gynoecium

arrest. The double mutant had altered auxin responsive gene expression. However, whole flower auxin levels did not change during flower maturation, suggesting that auxin might regulate flower maturation only under specific environmental conditions, or in localized organs or tissues of flowers. *arf6* and *arf8* single mutants and sesquimutants (homozygous for one mutation and heterozygous for the other) had delayed stamen development and decreased fecundity, indicating that ARF6 and ARF8 gene dosage affects timing of flower maturation quantitatively.

Key words: Auxin response factor, ARF, Auxin, Flower maturation, Jasmonate

## Introduction

During flowering plant fertilization, pollen is released from anthers and deposited on the stigma of a receptive gynoecium, where it germinates to produce pollen tubes that will deliver sperm to the ovules. In autogamous plants, pollination occurs more efficiently if the anther is close to the stigma. In several plants with mixed mating systems, heritable differences in anther-stigma distance influence rates of outcrossing versus selfing (Chang and Rausher, 1999; Karron et al., 1997; Motten and Stone, 2000). It is thus of interest to learn how flower development just prior to pollination is regulated and how male and female maturation are coordinated.

Transcription factors of the auxin response factor (ARF) family bind to auxin response elements (AuxREs, 5' tgtctc 3') present in promoters of numerous auxin-regulated genes, and ARFs mediate auxin-induced gene expression responses (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). Of the 22 predicted ARF proteins encoded in the *Arabidopsis* genome, five (MP/ARF5, ARF6, NPH4/ARF7, ARF8 and ARF19) have a glutamine-rich middle domain, and each of these can activate auxin-induced genes in transient expression

assays (Ulmasov et al., 1999a; Wilmoth et al., 2005). Mutations in *MP/ARF5*, *NPH4/ARF7*, *ARF8* and *ARF19* decrease auxin gene induction responses and cause auxin-related developmental defects at various stages of development. *mp/arf5* mutants have defects in embryonic, vascular and floral patterning (Aida et al., 2002; Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hardtke et al., 2004; Przemeczek et al., 1996). *nph4/arf7* mutants have defects in tropic growth of roots and hypocotyls, and *nph4/arf7 arf19* double mutants make very few lateral roots and have small leaves (Harper et al., 2000; Liscum and Briggs, 1996; Okushima et al., 2005; Stowe-Evans et al., 1998; Watahiki and Yamamoto, 1997; Wilmoth et al., 2005). Light-grown *arf8-1* mutant seedlings had elongated hypocotyls (Tian et al., 2004). Auxin regulates glutamine-rich ARF activity by promoting turnover of Aux/IAA proteins, which can interact with ARFs and inhibit gene induction (Gray et al., 2001; Kim et al., 1997; Tatematsu et al., 2004; Tian et al., 2003; Tian et al., 2002; Tiwari et al., 2003; Tiwari et al., 2001; Ulmasov et al., 1997; Zenser et al., 2001). Gain-of-function mutations in several different IAA genes encoding Aux/IAA proteins decrease

auxin-induced turnover of the corresponding proteins and cause phenotypes similar to those of loss-of-function *arf* mutants (Reed, 2001).

Phylogenetic analyses of *Arabidopsis* ARF proteins show that ARF6 and ARF8 form a clade and therefore may have overlapping functions (Remington et al., 2004). We have isolated plants with mutations in the *ARF6* and *ARF8* genes, and characterized phenotypes of single and double mutants. *arf6* and *arf8* single mutant plants have delayed stamen development and decreased fecundity, whereas *arf6 arf8* double mutant plants have a complete block in flower maturation. Decreased jasmonic acid (JA) production caused some aspects of this phenotype.

## Materials and methods

### Mutant isolation

To isolate T-DNA insertion mutations in *ARF6* (At1g30330) and *ARF8* (At5g37020), a T-DNA left border primer (JMLB: 5'-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3') and three degenerate primers that each hybridized to sequences encoding conserved domain IV of multiple ARF genes were used in PCR reactions to screen pools of genomic DNA representing 30,000 insertion mutants (<http://signal.salk.edu/tabout.html>). Individual plants carrying the *arf6-2* and *arf8-3* insertions were identified by PCR using one of these degenerate primers (ARF4IV3: 5'-CCATGGGTCATC(A/G)CCGAGGAGAAGAA(C/T)(A/G)TC-3') and the T-DNA left border primer. Sequencing these PCR products revealed that the 3' junction of the *arf6-2* insertion was 2301 nucleotides downstream of the start codon, and that the 3' junction of the *arf8-3* insertion was 2072 nucleotides downstream of the start codon (Fig. 1A). Absence of corresponding wild-type alleles in the insertion mutants was confirmed by Southern hybridization. A second primer upstream of the T-DNA insertion sites and specific to either *ARF6* (5'-GACGAATCTACTGCAGGAG-3') or *ARF8* (5'-CTAGATTCTGTTTCGTTGG-3') was used in combination with the degenerate primer to identify the wild-type alleles among progeny of crosses for linkage analysis and for constructing double mutants.

### Transgenic plants and genetics

For complementation, we cloned a 12.5 kb *Bam*HI genomic DNA fragment containing the complete open reading frame of *ARF6* (T4K22.6/At1g30330) from BAC T4K22 into pCAMBIA1300. This fragment extends 3262 bp upstream of the *ARF6* start codon and 5342 bp downstream of the stop codon, and also contains the C-terminal part of one other annotated open reading frame (predicted protein T4K22.7/At1g30320). We transformed the T-DNA carrying this construction from *Agrobacterium* strain GV3101 into *arf8-3* single mutant plants by vacuum infiltration (Bechtold et al., 1993), and identified six transformants whose self-progeny segregated 3:1 for hygromycin resistance encoded on the T-DNA. We fertilized each of these plants with *arf6-2 arf8-3* double mutant pollen (obtained after jasmonic acid treatment of mutant buds). Hygromycin-resistant F1 progeny of these crosses were allowed to self-fertilize, and the resulting F2 seed assayed for phenotype as presented in Table 3.

Hairpin RNA (*hpRNA*) constructs were cloned in the pB7GWIWG2(II) vector (Karimi et al., 2002). *ARF6 hpRNA* constructs had either nucleotides 28-581 or 1190-1771 of the *ARF6* open reading frame. The former of these covers a region with high similarity to *ARF8*, but did not apparently silence *ARF8*. The *ARF8 hpRNA* construct had nucleotides 1176-1663 of the *ARF8* open reading frame. For the construct with nucleotides 1190-1771 of the *ARF6*-coding region, when transformed into *arf8-3*, 50 T1 plants recapitulated the flower and leaf phenotypes of the *arf6-2 arf8-3* double mutant, 14 plants had an intermediate phenotype similar to the

sesquimutants, and six had no extra phenotype. In the wild-type background, three T1 plants had decreased fecundity, similar to *arf6-2*, and two had wild-type fecundity. In the *arf6-2* background, 27 had an *arf6*-like phenotype. For the construct with nucleotides 28-581 of the *ARF6*-coding region, when transformed into *arf8-3*, 62 plants recapitulated the flower and leaf phenotypes of the double mutant, 14 plants had an intermediate phenotype similar to the sesquimutants, and one had no extra phenotype. In the wild-type background, all 24 transformants had decreased fecundity, similar to *arf6-2*. In the *arf6-2* background, 51 had an *arf6*-like phenotype. For the construct with nucleotides 1176-1663 of the *ARF8*-coding region, when transformed into *arf6-2*, nine T1 plants recapitulated the flower and leaf phenotypes of the *arf6-2 arf8-3* double mutant, three plants had an intermediate phenotype similar to the sesquimutants, and 10 had no extra phenotype. In the wild-type background, 23 had decreased fecundity, similar to *arf8-3*, and four appeared similar to wild type. In the *arf8-3* background, 33 had an *arf8*-like phenotype. As none of the *hpRNA* constructs caused a phenotype resembling *arf6 arf8* double mutant plants when introduced into wild-type plants, we deduce that they each affected *ARF6* or *ARF8* but not both genes.

To make *ARF6* and *ARF8* promoter::GUS constructs, sequences up to and including the natural start codons were amplified by PCR and cloned upstream of the *GUS* start codon in a modified pPZP211 vector that contained GUS-nos and some upstream restriction sites derived from pEBGUS (Hagen et al., 1991). For *ARF6*, primers 5'-GCT-TAAGAATTAGCTGCAGAAACAAATGCTAGTTG-3' (*Pst*I site underlined) and 5'-CATGAGGTTGAGGATCCAACCCAGCTG-AAG (*Bam*HI site underlined) amplified a fragment including 2043 bp upstream of the start codon. For *ARF8*, primers 5'-GATTG-CGACGTAAGTGCAGGATATTACCATCG-3' (*Pst*I site underlined) and 5'-CACCTTCATGACCCTGTCGACCCAATCC-3' (*Sal*I site underlined) amplified a fragment that included 2387 bp upstream of the start codon. Constructs were transformed into ecotype Columbia plants. Multiple lines were analyzed and had similar staining patterns. Fig. 4 shows results from a representative line.

### Phenotypic analyses

Flower buds and dissected flower organs were measured using a camera lucida attachment on a dissecting microscope. For scanning electron microscopy, buds and flowers were fixed and processed as described (Laux et al., 1996), except that the tissue was dehydrated in an ethanol series (30%, 50%, 70%, 95%, 100%) instead of an acetone series. The specimens were imaged in a Cambridge S200 scanning electron microscope (LEO Electron Microscopy, Thornwood, NY) operated at 20 kV. Secondary electron images were acquired digitally using a 4pi image acquisition system (4pi Analysis, Durham NC).

For rescue of anther dehiscence, 4-5  $\mu$ l of linolenic acid [9(Z), 12(Z), 15(Z) octadecatrienoic acid, Cayman Chemical Company, Ann Arbor, MI; 25% stock solution in ethanol diluted to 0.1% in 0.1% Tween-20], OPDA (12-oxo-phytyldienoic acid, Cayman Chemical Company, 100 mg/ml stock solution in ethanol diluted to 100  $\mu$ M in 0.1% Tween-20) or JA (Sigma, 100  $\mu$ M or 500  $\mu$ M solution in 1% methanol 0.1% Tween-20) were applied to each flower bunch, and flowers observed 2-3 days later.

### Gene expression analyses

Total RNA was isolated from frozen tissues of 8-day-old seedlings or long-day-grown adult plants using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA gel blot hybridizations were performed as described (Nagpal et al., 2000). *ARF6* and *ARF8* probes were made from PCR products spanning *ARF6*- and *ARF8*-coding regions (amplified from cDNAs). For auxin and JA induction experiments, plants were sprayed with 10  $\mu$ M IAA or 500  $\mu$ M JA (in 1% methanol, 0.05% Tween-20) or with buffer alone, using a Preval sprayer (Precision Valve Corporation, Yonkers, NY). Gene probes were as described (Stintzi and Browse, 2000; Tian et al., 2002) and were labeled with  $^{32}$ P by random priming.

### Microarray gene expression analyses

Flower tissue was collected from *Arabidopsis thaliana* ecotype Columbia plants, *arf6-2/arf6-2 ARF8/arf8-3* plants, and *arf6-2 arf8-3* plants. Plants were grown for 6 weeks under a 16-hour light:8-hour dark regime. For the developmental time course, flowers were separated into stage 1-10 flowers, stage 11-12 flowers and stage 13-14 flowers. For auxin induction experiments, flower bunches containing flowers from stage 1 to stage 14 were used.

Tissue from approximately 40 plants was pooled for each RNA isolation and RNA from three biological replicates was pooled for cDNA synthesis. Total RNA (7 µg) was used to synthesize cDNA. A custom cDNA kit (Life Technologies, Grand Island, NY) was used with a T7-(dT)<sub>24</sub> primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit (Enzo Diagnostics, Farmingdale, NY). The cRNA was then fragmented in fragmentation buffer [5× fragmentation buffer: 200 mM Tris-acetate (pH 8.1), 500 mM KOAc, 150 mM MgOAc] at 94°C for 35 minutes before the chip hybridization. Fragmented cRNA (15 µg) was then added to a hybridization cocktail (0.05 µg/µl fragmented cRNA, 50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD* and *cre* hybridization controls, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100 mM MES, 1 M [Na<sup>+</sup>], 20 mM EDTA, 0.01% Tween 20). cRNA (10 µg) was used for hybridization in a volume of 200 µl per slide. ATH1 arrays (Redman et al., 2004) (Affymetrix, Santa Clara, CA) were hybridized for 16 hours at 45°C in the GeneChip Hybridization Oven 640 (Affymetrix). The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400 (Affymetrix) using wash protocol Eukge-ws2 version 4, and arrays were scanned with the Hewlett Packard model 2500 GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning and basic analysis. Data were scaled to a default target intensity of 500 before importing into Genespring 5.0 software. Sample quality was assessed by examination of 3' to 5' intensity ratios of certain genes.

Analysis of the Affymetrix gene chip data was carried out using Genespring 5.0 software. Raw data from each chip was normalized to the 50th percentile of measurements taken from that chip and genes were normalized to the median value. Following normalizations, pairwise comparisons of fold changes were carried out. For the auxin treatment experiment, data was restricted such that only genes with a raw data value greater than 100 and with an Affymetrix flag call of Present were considered. For the developmental time course experiment, data was restricted such that only genes with a raw data value greater than 300 and with a flag call of Present were considered. These raw cut-off levels were chosen as the expression level of genes below these numbers frequently had an Affymetrix flag call of Absent. Lists of genes presented in the supplementary data tables were derived by applying filter functions for threshold fold changes in gene expression. Gene expression data was also grouped using a self-organizing map using Genespring default parameters, with qualitatively similar conclusions.

We compared our microarray data to recently released data for floral stages 9, 12 and 15 (<http://www.weigelworld.org/resources/microarray/AtGenExpress>) (Schmid et al., 2005). In that dataset, among genes whose expression was referred to as 'present' and with a raw data value of at least 100, we identified 3141 genes with at least 2.5-fold differential expression at different stages. These genes included 1420 (83%) of the 1715 differentially expressed genes we identified. Taking into account the slightly different stages analyzed, these numbers suggest that the datasets are broadly consistent and our data are likely to be accurate for most genes.

The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through the following accession numbers: GSE2847, auxin induction in wild-type and *arf6 arf8* flowers (samples GSM62687-GSM62693); GSE2848, auxin response

factor-mediated flower gene expression (samples GSM62694-GSM62705); GSM62687, Columbia flowers\_stage 1-14 untreated; GSM62688, Columbia flowers\_stage 1-14\_30 minutes IAA treatment; GSM62689, Columbia flowers\_stage 1-14\_30 minutes mock treatment; GSM62690, *arf6/arf6 ARF8/arf8* flowers\_stage 1-14 untreated; GSM62691, *arf6/arf6 ARF8/arf8* flowers\_stage 1-14\_30 minutes IAA treatment; GSM62692, *arf6 arf8* flowers\_stage 1-14\_untreated; GSM62693, *arf6 arf8* flowers\_stage 1-14\_30 minutes IAA treatment; GSM62694, Columbia flowers\_stage 1-10; GSM62695, Columbia flowers\_stage 11-12; GSM62696, Columbia flowers\_stage 13-14; GSM62697, Columbia\_stem; GSM62698, *arf6/arf6 ARF8/arf8* flowers\_stage 1-10; GSM62699, *arf6/arf6 ARF8/arf8* flowers\_stages 11-12; GSM62700, *arf6/arf6 ARF8/arf8* flowers\_stages 13-14; GSM62701, *arf6/arf6 ARF8/arf8* \_stem; GSM62702, *arf6 arf8* flowers\_stage 1-10; GSM62703, *arf6 arf8* flowers\_stage 11-12; GSM62704, *arf6 arf8* flowers\_stage 13-14; GSM62705, *arf6 arf8* \_stem.

### Hormone measurements

Flower bunches were harvested into liquid nitrogen and kept frozen until analysis. Frozen flower tissue was stored at -80°C and transported on solid CO<sub>2</sub> (dry ice) to Lausanne for JA measurements and to St Paul for IAA analysis. Jasmonic acid was measured as described (Weber et al., 1997) with modifications described at <http://www.unil.ch/ibpv/WWWFarmer/WWWOxylipins/Docs/method.htm>.

IAA content was determined for triplicate samples weighing 50 to 110 mg FW (frozen weight). Purification and quantification of free IAA was based on the method described by Chen et al. (Chen et al., 1988) with modifications. Approximately 4 ml g<sup>-1</sup> FW extraction buffer [65% (v/v) isopropanol with 0.2 M imidazole (pH 7)] was added to each sample tube. Three tungsten carbide beads (3 mm; Qiagen, Valencia, CA) and ~40 ng g<sup>-1</sup> FW [<sup>13</sup>C<sub>6</sub>]IAA as an internal standard were also added to the sample tube before homogenization for 3 minutes at 15 Hz in a Mixer Mill MM 300 (Qiagen, Valencia, CA). After incubation on ice for 1 hour, samples were centrifuged (12,000 g for 5 minutes), and 50,000 dpm [<sup>3</sup>H]IAA was added to the supernatant as a radiotracer. The sample was diluted 10-fold with water and applied to a conditioned 50 mg NH<sub>2</sub> solid phase extraction (SPE) column (Varian, Walnut Creek, California). To condition the columns, 500 µl of hexane, acetonitrile, water, and 0.2 M imidazole (pH 7.0) were added sequentially followed by two water rinses of 1500 µl each. The loaded columns were washed sequentially with 500 µl each of hexane, ethyl acetate, acetonitrile and methanol, and 300 µl phosphoric acid. IAA was eluted in four additional 700 µl aliquots of phosphoric acid (PA). The pooled eluate was adjusted to pH 3 with 1 M succinic acid (SA; pH 6) in a ratio of PA:SA (v/v, 60:1).

IAA was further purified on a BSA column (Murphy, 1979; Schulze and Bandurski, 1979) made by linking BSA (Promega, Madison, Wisconsin) to Affiprep-10 (BioRad, Hercules, California) according to the manufacturer's protocol. Approximately 500 µl BSA-Affiprep was loaded onto empty SPE cartridges (Varian) and conditioned with PA:SA (3×500 µl). The pH-adjusted samples were loaded, and the column was washed with PA:SA (3 × 500 µl) followed by methanol (300 µl). IAA was eluted in five aliquots of methanol, 300 µl each. The pooled eluate containing free purified IAA was methylated by incubation with 1 ml ethereal diazomethane for approximately 5 minutes, evaporated to dryness under N<sub>2</sub>, and resuspended in 25 µl ethyl acetate. Quantification was by GC-MS-selected ion monitoring as described by Ribnicky et al. (Ribnicky et al., 1996) using a model 6890N GC/5973 Network MS (Agilent Technologies, Palo Alto, CA) equipped with an HP-5MS fused silica capillary column [30 m × 0.25 mm ID, (5%-phenyl)-methylpolysiloxane, 0.25 µm film thickness (Agilent Technologies)]. Injector temperature was set at 250°C and temperature programmed from 70°C (2 minutes) to 280°C at 20°C min<sup>-1</sup>. Ions at m/z 130, 136, 189 and 195 were monitored with dwell times of 50 ms per ion.

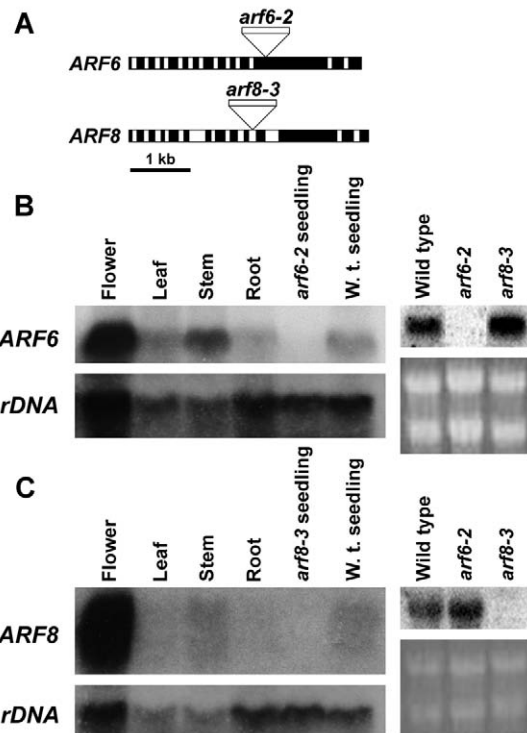
## Results

### *arf6* and *arf8* mutations affect self-fertilization quantitatively

We identified plants with mutations in *ARF6* or *ARF8* among a collection of T-DNA insertion mutants (Alonso et al., 2003). These *arf6-2* and *arf8-3* insertions each interrupted the transcribed sequence, and eliminated transcript of the corresponding gene (Fig. 1), suggesting that they were null mutations. *ARF6* and *ARF8* transcripts were each most abundant in flowers, and were expressed at lower levels in leaves, stems and roots (Fig. 1B,C) (Ulmasov et al., 1999b).

Roots and shoots of *arf6-2* and *arf8-3* single mutant and *arf6-2 arf8-3* double mutant seedlings closely resembled those of wild-type seedlings (data not shown). Other workers found that *arf8-1* mutant seedlings had a long hypocotyl (Tian et al., 2004). However, we observed a short hypocotyl in dark-grown *arf6-2*, *arf8-3* and *arf6-2 arf8-3* seedlings (Table 1). Primary inflorescence stems of adult *arf6-2* and *arf8-3* single mutant plants were 10-20% shorter than those of wild-type plants (Fig. 2A, Table 1), whereas numbers of flowers and of lateral branches were similar to those in wild-type plants (Table 1; data not shown). Thus, the short inflorescences of *arf6-2* and *arf8-3* plants arose from decreased internode elongation.

Flowers of *arf6-2* and *arf8-3* mutant plants had normal organ number and position, but often produced no seed (Table 1, Fig. 2A). Prior to flower stage 12 (Smyth et al., 1990), when buds reached 2 mm long, mutant flowers appeared normal and they were present in normal numbers (Table 1). However, at stage 12 development of mutant stamens lagged relative to development of other flower parts. Stamen filaments of stage 12 *arf6-2* and *arf8-3* flowers were shorter than those of stage 12 wild-type flowers, and petals of *arf6-2* flowers were also shorter than those of wild-type flowers (Fig. 3, left). *arf6-2* anthers dehiscence to release pollen slightly later than did wild-type anthers relative to the youngest bud of 2 mm or longer (Table 1). *arf8-3* anthers also dehiscence slightly later than did wild-type anthers, although not statistically significantly (Table 1). Mutant stamen filaments did elongate further as the flowers grew older. At the time when their anthers dehiscence (anthesis), *arf6-2* and *arf8-3* mutant stamens were roughly as long as wild-type stamens had been at anthesis, but by then *arf6-2* and *arf8-3* carpels had already grown slightly longer than the stamens (Fig. 3, right panel). These results indicate



**Fig. 1.** T-DNA insertion mutations in *ARF6* and *ARF8*. (A) Locations of T-DNA insertions. Horizontal bars represent *ARF6* (At1g30330) and *ARF8* (At5g37020) -coding regions, with exons (black) and introns (white) indicated. (B) RNA gel blot hybridization using *ARF6* and *rDNA* probes. (Left panel) RNA from 8-day-old wild-type seedlings, *arf6-2* mutant seedlings or wild-type flowers, stems, leaves or roots. (Right panel) RNA from wild-type or mutant flowers. (C) RNA gel blot hybridization using *ARF8* and *rDNA* probes. Left panel, RNA from wild-type seedlings, *arf8-3* mutant seedlings or wild-type flowers, stems, leaves or roots. (Right panel) RNA from wild-type or mutant flowers.

that *arf6-2* and *arf8-3* plants self-pollinated inefficiently in part because the anthers were too far from the stigma, as a consequence of slightly delayed filament elongation and anther dehiscence.

Wild-type plants carrying hairpin RNA constructs that target gene silencing of either *ARF6* or *ARF8* also had reduced seed

**Table 1. Measurements of wild-type and mutant hypocotyls, inflorescences and flowers\***

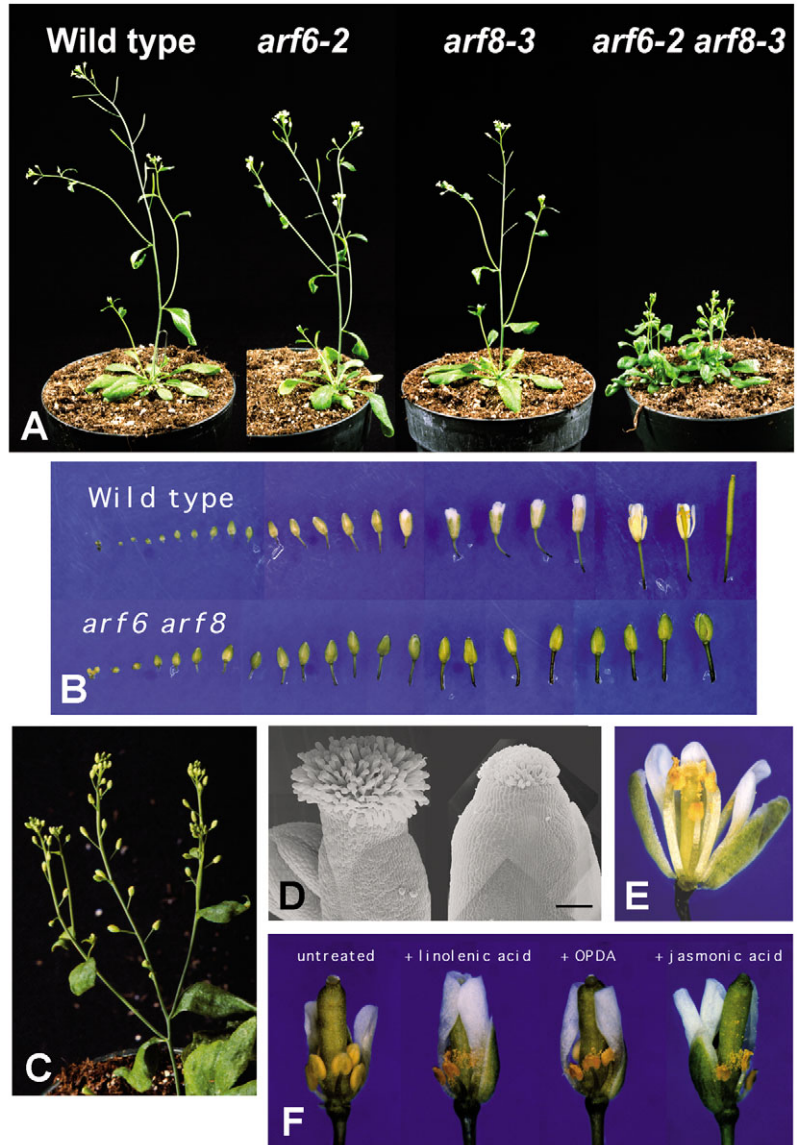
	Genotype					
	<i>ARF6/ARF6</i> <i>ARF8/ARF8</i>	<i>arf6-2/arf6-2</i> <i>ARF8/ARF8</i>	<i>ARF6/ARF6</i> <i>arf8-3/arf8-3</i>	<i>arf6-2/arf6-2</i> <i>ARF8/arf8-3</i>	<i>ARF6/arf6-2</i> <i>arf8-3/arf8-3</i>	<i>arf6-2/arf6-2</i> <i>arf8-3/arf8-3</i>
Hypocotyl length in D, 4 days (mm)	12.5±1.2 (31)	9.7±1.4 (24) <sup>†</sup>	11.2±1.1 (23) <sup>†</sup>			5.1±0.6 (13) <sup>†</sup>
Inflorescence length 26 days (cm)	9.9±1.9 (35)	8.1±2.0 (28) <sup>†</sup>	7.7±2.4 (28) <sup>†</sup>	7.7±1.6 (19) <sup>†</sup>	6.9±1.0 (26) <sup>†</sup>	1.7±0.5 (19) <sup>†</sup>
Inflorescence length, 45 days (cm)	15.6±2.3 (7)	13.1±3.0 (7)	10.9±2.0 (7) <sup>†</sup>			4.0±1.2 (9) <sup>†</sup>
Number of inflorescence stems <sup>‡</sup>	13.5±2.6 (13)	12.4±1.9 (7)	14.6±2.9 (7)			11.0±2.2 (8) <sup>†</sup>
Proportion fertile flowers <sup>‡</sup>	0.84±0.12 (13)	0.15±0.08 (14) <sup>†</sup>	0.24±0.11 (14) <sup>†</sup>	0±0 (6) <sup>†</sup>	0.07±0.07 (9) <sup>†</sup>	0±0 (9) <sup>†</sup>
Number of buds on primary inflorescence <sup>‡</sup> :						
smaller than 1 mm long	11.7±1.0 (7)	9.9±1.1 (7) <sup>†</sup>	9.4±1.7 (7) <sup>†</sup>			10.8±1.1 (8)
1-2 mm long	7.7±0.7 (7)	7.3±1.2 (7)	7.4±0.5 (7)			7.1±1.1 (8)
from 2 mm bud to anthesis	1.6±0.9 (7)	3.7±1.3 (7) <sup>†</sup>	2.1±0.8 (7)			(no anthesis)

\*Data are mean±s.d. (n).

<sup>†</sup>Values that were significantly different from wild-type values by *t*-test (P<0.05).

<sup>‡</sup>Data for number of inflorescence stems, proportion of fertile flowers and number of buds of different size classes per primary inflorescence were measured on 33-day-old plants also used for the organ length data presented in Fig. 3.

**Fig. 2.** Phenotypes of *arf6-2*, *arf8-3* and *arf6-2 arf8-3* double mutant plants. (A) 47-day-old plants. (B) Flowers of wild-type and *arf6-2 arf8-3* double mutant plants. Flower buds from a single inflorescence are arranged from youngest to oldest. (C) Inflorescence stem of an *arf6-2 arf8-3* plant showing arrested flower buds. (D) Scanning electron micrographs of apices of gynoecia of mature wild-type (left) and arrested *arf6-2 arf8-3* (right) flowers of the same age. Scale bar: 0.1 mm. (E) Mature wild-type flower at the stage of self-fertilization. (F) *arf6-2 arf8-3* flower bud untreated (left), or 2-3 days after application of linolenic acid, OPDA or jasmonic acid. In E and F, buds were dissected to reveal internal organs.



set (see Materials and methods), confirming that deficiency of either *ARF6* or *ARF8* reduces self-fertilization. *arf6-2/arf6-2 ARF8/arf8-3* and *ARF6/arf6-2 arf8-3/arf8-3* plants, with just one wild-type copy of either *ARF8* or *ARF6*, had elongated inflorescence stems and open flowers, but had short stamen filaments and even fewer fruits with seed than did *arf6-2* or *arf8-3* single mutant plants (Table 1). We refer to *arf6-2/arf6-2 ARF8/arf8-3* and *ARF6/arf6-2 arf8-3/arf8-3* plants as sesquimutants (Latin ‘sesqui-’ meaning one and a half, as in ‘sesquicentennial’). Sesquimutant anthers dehisced to release pollen, and after manual self-pollination these flowers produced abundant seed, suggesting that for sesquimutants as for single mutants, decreased self-pollination arose from delayed filament elongation and anthesis.

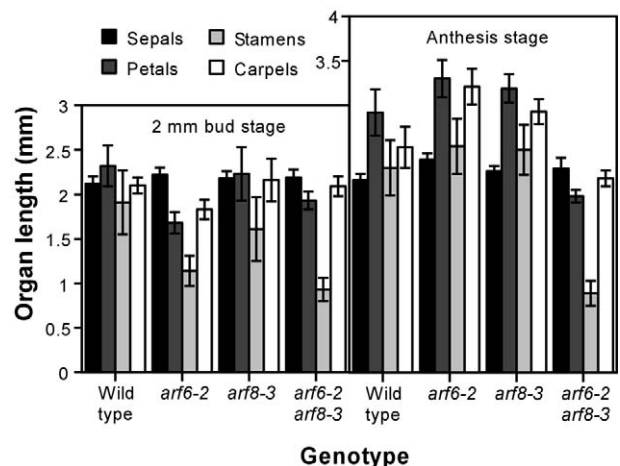
The decreased seed production in *arf6-2*, *arf8-3* and both sesquimutant plants indicates that *ARF6* and *ARF8* have similar functions in stamen development, and that *ARF6* and *ARF8* gene dosage has a quantitative effect on rates of self-pollination. Moreover, a genomic *ARF6* transgene could rescue the decreased fecundity of the *arf8-3* mutant (Table 2), indicating that extra copies of *ARF6* can substitute for absence of *ARF8*.

**ARF6 and ARF8 are required for flower maturation**

*arf6-2 arf8-3* double mutant plants were identified among self-progeny of sesquimutant plants. Double mutant seedlings had normal root and leaf growth, and appeared normal during the early part of vegetative growth. Beginning at the time of flowering, double mutant leaves grew unevenly and twisted instead of lying flat. Upon flowering the inflorescence stems elongated less than those of wild-type or single mutant plants (Table 1, Fig. 2A). Most strikingly, flowers of *arf6-2 arf8-3*

double mutant plants failed to open or to produce seed. Wild-type flowers open at stage 13, at which point petals, stamen filaments, and carpels are elongating, anthers are about to

**Fig. 3.** Lengths of wild-type, *arf6-2*, *arf8-3* and *arf6-2 arf8-3* floral organs  $\pm$ s.d. Data were gathered from flower bunches of the main inflorescence stem from seven 33-day-old plants of each genotype. The left panel shows organ lengths of the youngest flower bud of 2 mm length. The right panel shows organ lengths of the youngest flowers that were releasing pollen (anthesis). For the *arf6-2 arf8-3* double mutant (which does not undergo anthesis), organs of the fourth flower after the youngest 2 mm bud were measured. For the other genotypes, numbers of flowers of different size classes are indicated in Table 1.



**Table 2. Rescue of *arf8-3* fecundity by genomic *ARF6* transgene\***

Genotype	Proportion of flowers giving seed <sup>†</sup>
Wild type	0.62±0.14 (14)
<i>arf6-2</i>	0.16±0.10 (14)
<i>arf8-3</i>	0.24±0.12 (12)
<i>arf8-3 gARF6</i> <sup>‡</sup>	0.72±0.17 (39)

\*Data are mean±s.d. (n).

<sup>†</sup>For *arf8-3 gARF6*, data are pooled from T2 progeny of six transformants that segregated roughly 3:1 for hygromycin-resistance, indicating a single transgene locus.

dehisce, and carpels become fully competent to receive pollen (Fig. 2E). By contrast, *arf6-2 arf8-3* mutant flowers arrested at stage 12 as infertile closed buds (Fig. 2B,C). Petals, stamen filaments and carpels each ceased to elongate (Fig. 3). Epidermal cells of double mutant stamen filaments were about half as long as those of wild-type filaments (13 compared with 24 μm), indicating that decreased cell expansion caused the short filaments. Anthers failed to dehisce and release pollen grains visible within the locules (Fig. 2F, left panel). In the carpels, the mutant stigmatic papillae were shorter than in wild-type flowers (Fig. 2D). Wild-type pollen could germinate on these stigmas, and pollen tubes grew to a limited extent. However, fertilization occurred at much lower frequency than after wild-type stigmas were manually pollinated, and very few seed formed in ovaries of such manually fertilized *arf6-2 arf8-3* carpels.

Among self-progeny of sesquimutants, all *arf6-2 arf8-3* double mutant plants had twisted leaves, short inflorescence stems and closed flower buds, and produced no seed; all sesquimutant plants had open flowers but produced almost no seed; and all *arf6-2* and *arf8-3* single mutants produced seed (Table 3). Thus, closed flower buds and infertility were completely linked to both the *arf6-2* and *arf8-3* mutations. In addition, a transgene carrying the genomic *ARF6* gene rescued both the strong phenotype of double mutants and the reduced fecundity of sesquimutants (Table 3). Hairpin RNA (hpRNA) constructs (Smith et al., 2000) designed to silence specifically *ARF6* or *ARF8* recapitulated the double mutant phenotypes. Thus, *arf6-2* mutant plants carrying an *hpARF8* transgene or *arf8-3* plants carrying an *hpARF6* transgene resembled *arf6-2*

*arf8-3* double mutant plants (see Materials and methods). Finally, we isolated three new *arf8* alleles in a screen for enhancers of *arf6-2*, and these double mutants had the same phenotypes as *arf6-2 arf8-3* plants (P. H. Reeves and J.W.R., unpublished).

### ***ARF6* and *ARF8* are expressed in multiple flower organs**

Microarray data indicate that *ARF6* and *ARF8* are expressed at multiple floral stages in all flower organs (Schmid et al., 2005) (see below). To examine in more detail where *ARF6* and *ARF8* may be expressed, we fused 2.1 kb of *ARF6* and 2.4 kb of *ARF8* promoter sequences to the *GUS* reporter gene and examined X-gluc staining in transgenic plants carrying these fusions. Both fusions were expressed in flowers at multiple stages. Fig. 4 shows staining patterns at stages 11, 12 and 13, spanning the period of arrest of double mutant flowers. For the *P<sub>ARF6</sub>::GUS* fusion, sepals had staining at all stages of flower development (Fig. 4A-D). Petals stained strongly at flower stages 9-10 (data not shown) and this petal staining decreased at stage 11 (Fig. 4A) and disappeared after flower stage 12. In anthers, staining appeared at stage 11 in the tapetum (Fig. 4A,H), then disappeared early in stage 12 when the tapetum degrades (Sanders et al., 1999) (Fig. 4B) and reappeared throughout the anther late in stage 12 (Fig. 4C). Anther staining persisted at least until stage 13. In stamen filaments, staining appeared at stage 11-12, and persisted through stage 13, especially near the apical end of the filament (Fig. 4B-D). Staining appeared throughout the gynoecium at early stages up to stage 12, and was especially strong in ovules (Fig. 4A-C). Gynoecium staining weakened somewhat late in stage 12, but persisted through stage 13, especially near the apical end including the style (Fig. 4D).

The patterns for *P<sub>ARF8</sub>::GUS* (Fig. 4E-G,I) were similar to those for *P<sub>ARF6</sub>::GUS*. *P<sub>ARF8</sub>::GUS* staining appeared in sepals at all stages, and in petals at stages 9-10. Anthers stained in the tapetum at stage 11 (Fig. 4E,I), and throughout the anther at stages 12-13 (Fig. 4F,G). Filament staining appeared at stage 12 and persisted through stage 13 (Fig. 4F,G). Gynoecia stained throughout at stage 11, and this staining decreased at stages 12-13 (Fig. 4E-G).

These expression patterns correlate with the timing of anther and gynoecium arrest in *arf6-2 arf8-3* double mutant flowers,

**Table 3. Segregation of floral fecundity phenotypes among self-progeny of plants carrying *arf6-2* and *arf8-3* mutations, with and without a segregating genomic *ARF6* transgene (*gARF6*)**

Self-fertilized parent	n	Fecund*	Semi-fecund <sup>†</sup>	Infertile <sup>‡</sup>	χ <sup>2</sup> (1:2:1)
<i>arf6-2/arf6-2 arf8-3/ARF8</i> <sup>§</sup>	119	22 (18%)	63 (53%)	34 (29%)	2.8 (P>0.1)
<i>arf6-2/ARF6 arf8-3/arf8-3</i> <sup>§</sup>	193	51 (26%)	93 (48%)	49 (25%)	0.3 (P>0.75)
<i>arf6-2/ARF6 arf8-3/arf8-3 gARF6</i> , all progeny <sup>¶</sup>	350	249 (71%)	82 (23%)	19 (5%)	401 (P<0.005)
<i>arf6-2/ARF6 arf8-3/arf8-3 gARF6</i> , Hyg <sup>R</sup> progeny <sup>¶</sup>	213	188 (88%)	25 (12%)	0 (0%)	457 (P<0.005)

\*Fecund individuals produced abundant seed, although in many cases less than did wild-type plants.

<sup>†</sup>Semi-fecund plants had open flowers but only very few flowers produced seed.

<sup>‡</sup>Infertile plants had twisted leaves, closed flower buds and short inflorescence stems as in Fig. 2C, and produced no seed.

<sup>§</sup>Genotypes of the segregating locus in each plant among self-progeny of *arf6-2/arf6-2 arf8-3/ARF8* and *arf6-2/ARF6 arf8-3/arf8-3* plants were determined using a PCR assay (see Materials and methods). In all 312 plants, fecund individuals were homozygous for the wild-type allele (*ARF8/ARF8* or *ARF6/ARF6*, respectively), semi-fecund individuals were heterozygous (*arf8-3/ARF8* or *arf6-2/ARF6*) and infertile individuals were homozygous mutant.

<sup>¶</sup>Data for *arf6-2/ARF6 arf8-3/arf8-3 gARF6* self-progeny are pooled data representing six *gARF6* lines. The low P values for the 1:2:1 null hypothesis reflect a lower than expected frequency of semi-fertile and infertile plants in populations segregating for both *arf6-2* and *gARF6*, showing that the transgene rescued these phenotypes.

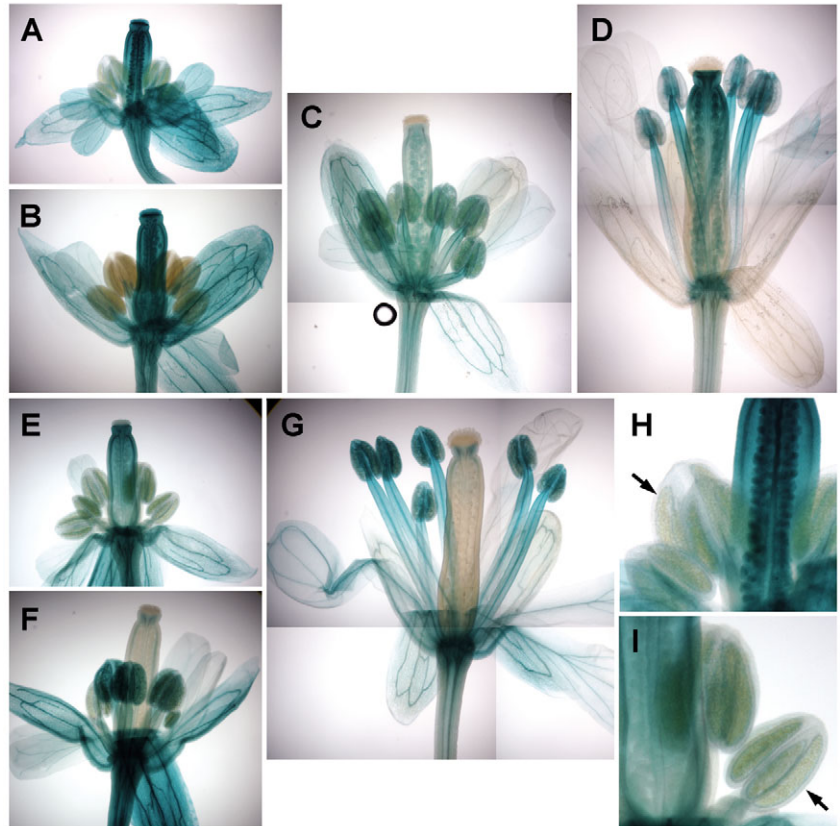
Hyg<sup>R</sup>, hygromycin-resistant self-progeny that carry the *gARF6* transgene.

and suggest that ARF6 and ARF8 are active in anthers and filaments at stage 12 and in gynoecia and ovules at stages 11 and 12. Nevertheless, additional factors such as the microRNA *miR167*, which targets *ARF6* and *ARF8* transcripts (Allen et al., 2005; Kasschau et al., 2003), or other post-transcriptional effects, may also influence the timing and location of ARF6 and ARF8 activity in flowers.

### Gene expression changes in *arf6 arf8* flowers

To identify regulatory changes that occur during flower maturation, we used Affymetrix microarrays (Redman et al., 2004) to assess global gene expression patterns in wild-type, sesquimutant (*arf6-2/arf6-2 arf8-3/ARF8*) and *arf6-2 arf8-3* double mutant flowers. We isolated RNA from pooled flower buds of stages 1-10 (closed buds shorter than 2 mm, also containing a small amount of stem tissue), stages 11-12 (unopened/opening, corresponding to the stage at which *arf6-2 arf8-3* flowers arrest), or stages 13-14 (flowers open but organs not yet fallen off), as well as the tops of the inflorescence stem bearing these flowers (Smyth et al., 1990). As *arf6-2 arf8-3* double mutant flowers do not open, for stage 13-14 double mutant flowers we used unopened buds immediately beneath the youngest stage 11-12 flowers, in the position where stage 13-14 flowers would have been in wild-type inflorescences. In wild-type flowers, of the 12,300 genes with robust expression levels (see Materials and methods), 1715 differed at least 2.5-fold in expression levels between different floral stages. Table S1 (in the supplementary material) lists these genes and their expression levels at each stage in the three genotypes. Three-hundred and eighty-seven of these genes were expressed at their highest level in stage 1-10 flowers, 417 were expressed at their highest level at stages 11-12, and 911 were expressed at their highest level at stages 13-14 (Fig. 5A). Five-hundred and ninety-one were also expressed in stems. One-thousand four-hundred and twenty (83%) of these genes also had stage-specific expression in a recent study that included floral stages 9, 12 and 15 (Schmid et al., 2005). These results indicate that roughly 14% of expressed genes in flowers change in expression level according to developmental stage.

Gene expression in *arf6-2 arf8-3* double mutant flower buds was substantially different. Nine-hundred and forty-two genes were expressed at lower level in double mutant than wild-type flowers at one or more stages, including both 617 developmentally regulated and 325 non-developmentally regulated genes (see Table S2 in the supplementary material, Fig. 5B), and 602 genes were expressed at higher level in double mutant than wild-type flowers at one or more stages (Fig. 5C). Of the 1715 developmentally regulated genes we identified in wild-type flowers, 718 (42%) were no longer differentially regulated at different floral stages in the double mutant (Fig. 5A; see Table S1 in the supplementary material).



**Fig. 4.** Expression of *P<sub>ARF6</sub>::GUS* and *P<sub>ARF8</sub>::GUS* fusions in flowers. (A-D,H) *P<sub>ARF6</sub>::GUS* flowers. (E-G,I) *P<sub>ARF8</sub>::GUS* flowers. (A) Stage 11. (B,C) Stage 12. (D) Stage 13. (E) Stage 11. (F) Stage 12. (G) Stage 13. (H,I) Higher magnification of images in A and E. Arrows indicate tapetum staining.

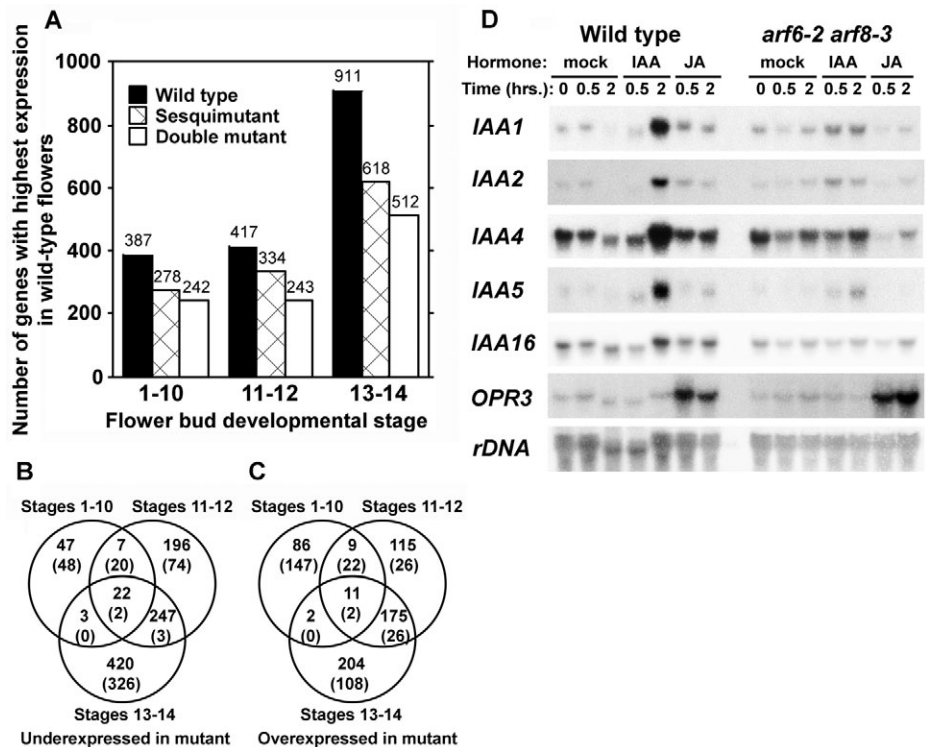
Moreover, 160 additional stage 13-14 genes were still developmentally regulated in the double mutant before stages 11-12 but no longer increased at stages 13-14 as they did in wild-type flowers. These results indicate that ARF6 and ARF8 control, directly or indirectly, roughly one in 13 genes that are expressed in flowers, and roughly half of developmentally regulated genes in opening flowers. Similar proportions of stage 1-10, stage 11-12, and stage 13-14 genes (defined based on the stage of highest expression in wild-type flowers) were affected (Fig. 5A).

In the *arf6-2/arf6-2 arf8-3/ARF8* sesquimutant, 473 genes were underexpressed and 331 genes were overexpressed at one or more stages compared to wild-type flowers (Fig. 5B,C). Among the developmentally regulated genes in wild-type flowers, 485 (28%) of the genes were no longer differentially regulated at different floral stages (Fig. 5A). The intermediate number of genes affected in the *arf6-2/arf6-2 arf8-3/ARF8* sesquimutant shows that *ARF6* and *ARF8* gene dose affects the global gene expression pattern quantitatively, consistent with the intermediate phenotype of sesquimutant flowers.

### *arf6 arf8* double mutant flowers have decreased auxin response

ARF6 and ARF8 can mediate auxin-induced gene activation (Ulmasov et al., 1999a). To determine whether altered auxin response might underlie the developmental arrest in *arf6-2*

**Fig. 5.** Effects of *arf6-2* and *arf8-3* mutations on gene expression in flowers. (A) Numbers of genes with expression that differs by at least 2.5-fold between different stages in wild-type flowers, grouped by stage of highest expression, together with numbers of genes in each wild-type expression class that were also developmentally regulated in *arf6-2/arf6-2 arf8-3/ARF8* sesquimutant and *arf6-2 arf8-3* double mutant flowers. (B,C) Venn diagrams showing numbers of genes that were underexpressed (B) or overexpressed (C) at different stages in *arf6-2 arf8-3* double mutant flowers, and in *arf6-2/arf6-2 arf8-3/ARF8* sesquimutant flowers (in parentheses). (D) Gene expression in response to auxin and jasmonic acid. Wild-type or *arf6-2 arf8-3* mutant mature flowering plants were mock-treated or treated with IAA or JA for the times indicated, and blots of RNA from inflorescence apices were hybridized with the indicated probes.



*arf8-3* flower development, we analyzed global gene expression responses to exogenous auxin in wild-type and mutant whole floral apices using Affymetrix microarrays (see Materials and methods). Expression of 35 genes was increased at least twofold in wild-type floral apices after a 30 minute auxin treatment but changed less than 1.5-fold after a mock treatment (Table S3 in the supplementary material). Of these 35 genes, just 23 responded to auxin in the sesquimutant and 14 responded to auxin in the double mutant. Of the 21 genes that auxin induced in wild-type but not double mutant floral apices, seven (*SAUR62*, *SAUR63*, *SAUR64*, *SAUR65*, *SAUR67*, *SHY2/IAA3* and *IAA4*) were underexpressed in the double mutant at one or more stages in the absence of exogenous auxin and were developmentally regulated at different stages in wild-type flowers (see Tables S1, S2, S3 in the supplementary material). RNA blot hybridization experiments that included a 2-hour time point also showed a decrease in auxin induction in the double mutant – treatment with exogenous auxin induced the *IAA1*, *IAA2*, *IAA4*, *IAA5* and *IAA16* genes in flowers of wild-type plants, but induced these genes much less in *arf6-2 arf8-3* flowers (Fig. 5D). These data indicate that ARF6 and ARF8 are required for maximal auxin response in developing flowers, and raise the possibility that auxin induces flower

maturation. We also observed that auxin induced 165 genes in double mutant flowers that it did not induce in wild-type flowers. This finding suggests that flower arrest may affect auxin response secondarily, and that other ARFs are active in *arf6-2 arf8-3* double mutant flowers.

To assess whether an increase in auxin level in flowers might regulate flower maturation, we measured levels of auxin in the same staged buds used for the developmental time course microarray experiments. As shown in Table 4, in wild-type flower buds auxin levels did not change appreciably with developmental stage. Levels were higher in double mutant than wild-type flowers, especially at stages 1-10. This may be an indirect regulatory effect of decreased auxin response, as other auxin-resistant mutants (such as *axr1*) have increased auxin levels (Nordstrom et al., 2004), and ARF8 has previously been suggested to regulate seedling auxin levels by inducing genes encoding auxin conjugation enzymes (Tian et al., 2004). Thus, an overall increase in free auxin in flowers probably does not induce flower maturation under the growth conditions used.

### Decreased jasmonic acid production accounts for a subset of *arf6 arf8* phenotypes

Other workers have found that flower buds of mutants deficient

**Table 4. Levels of IAA and JA in floral buds of different genotypes**

Flower stage	Concentration of IAA (ng/g frozen weight)±s.d.*			Concentration of JA (pmole/g frozen weight)±s.d.*		
	Wild type	<i>arf6-2/arf6-2 ARF8/arf8-3</i>	<i>arf6-2 arf8-3</i>	Wild type	<i>arf6-2/arf6-2 ARF8/arf8-3</i>	<i>arf6-2 arf8-3</i>
1-10	59.6±11.5	67.9±9.9	117.6±14.4	29.5±1.9	20.0±11.0	0 <sup>†</sup>
11-12	42.0±2.4	64.7±19.5	81.0±3.8	196.2±14.2	63.1±4.4	0 <sup>†</sup>
13-14	54.2±5.5	44.8±4.0	71.4±9.8	52.3±1.9	14.1±7.6	0 <sup>†</sup>

\*In each case values are means of three measurements±s.d.

<sup>†</sup>0 indicates JA concentration below the detection limit.



in synthesis of the growth regulator jasmonic acid (JA) opened more slowly than did wild-type buds, and anthers of these mutant flowers failed to dehisce, although the flowers were female fertile (Ishiguro et al., 2001; Sanders et al., 2000; Stintzi and Browse, 2000). These observations suggested that the *arf6-2 arf8-3* phenotypes may be related to decreased JA production or response. To test this idea, we measured JA levels in wild-type and mutant flowers, and we assessed whether JA could rescue aspects of the *arf6-2 arf8-3* double mutant phenotype.

We measured JA levels in the same staged flower buds used for the developmental time course microarray experiment. In wild-type flowers, JA levels increased 6.7-fold between stage 1-10 and stage 11-12 buds, and then decreased between stages 11-12 and 13-14 (Table 4). The peak of JA production at stages 11-12, just before anther dehiscence and bud opening, coincides with the timing of the JA requirement revealed by phenotypes of JA-deficient mutants.

The *arf6-2 arf8-3* double mutant flowers had a JA level below the detection limit at all stages (Table 4), indicating that normal JA production in flowers requires ARF6 and ARF8. This activity was specific to flowers, as *arf6-2 arf8-3* leaves had wild-type JA levels (data not shown). Sesquimutant flowers, which do not arrest at stage 12, also had a peak JA level at stages 11-12, but had lower JA levels than wild-type flowers at all stages. This result suggests that the level of ARF6 and ARF8 determines the level of JA production, consistent with the possibility that ARF6 and ARF8 regulate one or more genes required for JA production. Several JA biosynthetic pathway genes were underexpressed in double mutant stage 11-12 buds (Table S1 in the supplementary material), including *LOX2* encoding a lipoxygenase (At3g45140, 1.9-fold higher in wild-type than double mutant stage 11-12 buds), *AOS* encoding allene oxide synthase (At5g42650, 2.7-fold higher) and *OPR3* encoding OPDA reductase (At2g06050, 3.2-fold higher). The promoters of *LOX2* and *AOS* each have two AuxRE motifs within 40 bp of each other and the promoter of *OPR3* has two pairs of AuxRE motifs, suggesting that ARF6 and ARF8 might bind directly to these promoters. However, although auxin can induce *LOX2* and *AOS* in seedlings (Tiryaki and Staswick, 2002), these genes were not induced by auxin in our experiment (see Table S3 in the supplementary material). Moreover, as JA also induces these genes (Bell and Mullet, 1993; Costa et al., 2000; Kubigsteltig et al., 1999) these data do not distinguish whether ARF6 and ARF8 induce these genes directly, or whether the higher JA level in wild-type buds induces them. Indeed, exogenous JA induced the JA-responsive *OPR3* gene normally in *arf6-2 arf8-3* mutant flowers (Fig. 5D), indicating that double mutant flowers could respond to JA, and suggesting that a low JA level could have caused the decreased *OPR3* expression in the double mutant. The *DADI* gene encoding a phospholipase required for JA production is expressed in stamen filaments beginning at stage 12 (Ishiguro et al., 2001), suggesting that it could be a target of ARF6 and ARF8. However, we have failed to find evidence for altered *DADI* level in *arf6-2 arf8-3* flowers (data not shown).

JA feeding experiments revealed that the decrease in JA production contributed to the *arf6-2 arf8-3* mutant phenotype. Exogenous JA induced *arf6-2 arf8-3* anthers to dehisce (Fig. 2F). When pollen released by anthers of JA-treated *arf6-2 arf8-3* flowers was used to fertilize gynoecia of wild-type plants,

viable seed were produced, indicating that this JA-rescued pollen was functional. Exogenous JA also increased *arf6-2 arf8-3* petal elongation slightly and caused slight but incomplete flower bud opening. By contrast, JA did not rescue the short filaments of *arf6-2 arf8-3* stamens, or the developmental defects in carpels. Manual self-pollination of JA-treated *arf6-2 arf8-3* double mutant plants produced only a small number of seed. Siliques containing these seed often remained green, and the seed were slow to mature and the embryos sometimes had abnormalities such as fused cotyledons. These results suggest that the decreased JA content of double mutant flowers caused the anther dehiscence defect, but did not cause the stamen filament elongation or gynoecium maturation defects. The JA biosynthetic precursors linolenic acid (18:3) and OPDA also induced anther dehiscence (Fig. 2F), whereas other fatty acids [palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2)] did not. Thus, the regulated step may be upstream of linolenic acid. However, the positive feedback of JA synthesis means that these feeding experiments cannot determine the regulated step unambiguously.

## Discussion

ARF6 and ARF8 regulate multiple events including inflorescence stem elongation, stamen filament elongation, anther dehiscence, stigmatic papillae elongation, gynoecium maturation and flower bud opening. Loss of *ARF6* or *ARF8* in single mutants caused delayed stamen filament elongation and delayed anther dehiscence leading to decreased self-pollination, whereas loss of both genes in the *arf6-2 arf8-3* double mutant caused complete male and female infertility. ARF6 and ARF8 are thus essential for *Arabidopsis* reproduction. Moreover, they coordinate both stamen and gynoecium maturation at stage 12, thereby allowing flowers to self-fertilize efficiently. Numerous monocot and dicot species have orthologs of ARF6 and ARF8 (<http://www.tigr.org/tdb/tgi/>) (Remington et al., 2004), and these may similarly regulate flower maturation in other plants.

Phenotypes of *arf6-2* and *arf8-3* single mutants, sesquimutants and *arf8-3 gARF6* plants indicate that ARF6 and ARF8 normally act partially redundantly, and that *ARF6* and *ARF8* gene dosage affects fecundity quantitatively. ARF6 and ARF8 have very similar DNA-binding and dimerization domains (Remington et al., 2004; Ulmasov et al., 1999a), and *P<sub>ARF6</sub>::GUS* and *P<sub>ARF8</sub>::GUS* have very similar expression patterns. It therefore seems likely that ARF6 and ARF8 each regulate common promoters, either as homodimers or heterodimers. They diverge significantly in the Q-rich middle domains, which are required for gene activation. Apparently, this divergence does not confer distinct developmental functions on ARF6 and ARF8, although it might affect the strength of their activity.

## Possible regulatory targets of ARF6 and ARF8

The microarray expression data indicate that *arf6-2* and *arf8-3* mutations together have a large effect on gene expression at all three developmental stages we analyzed. However, the most relevant regulatory targets may be those that are misexpressed in stage 11-12 buds, when *arf6-2 arf8-3* double mutant flowers arrested. As ARF6 and ARF8 are transcriptional activators, the 472 genes underexpressed at this stage are more likely to be

direct targets. Perhaps the strongest candidates for direct ARF6 and ARF8 targets are the seven genes that were auxin-induced in wild-type but not double mutant flowers, developmentally regulated in wild-type flowers, and underexpressed in double mutant buds (see Table S3 in the supplementary material). These genes include five *SAUR* genes from a single clade, and the sister genes *SHY2/IAA3* and *IAA4*. The functions of SAUR proteins are unknown. IAA3 and IAA4 may dimerize with ARF6 and ARF8 to inhibit gene activation activity, and may constitute a negative feedback loop.

Many genes were expressed at intermediate level in sesquimutant flowers and therefore may be regulated by ARF6 and ARF8 protein concentration. These genes might regulate those phenotypes that ARF6 and ARF8 affect quantitatively, such as stamen development and inflorescence stem elongation. Of the 472 genes that were underexpressed at stages 11-12 in the double mutant, 168 were expressed in the sesquimutant at less than two-thirds (67%) of wild-type level, and are therefore candidates by this criterion. Conversely, genes that were expressed in the sesquimutant at a level close to the level in wild-type flowers are more likely to regulate phenotypes that are not obviously quantitative such as gynoecium maturation, or to have been affected secondarily by the arrest of double mutant flowers at stage 12. These genes might also be regulated preferentially by ARF8 rather than ARF6, and respond to the ARF8 supplied by the single wild-type *ARF8* allele present in the sesquimutant.

Phenotypes of *arf6-2 arf8-3* flowers and the expression patterns of *ARF6* and *ARF8* suggest that ARF6 and ARF8 regulate target genes in all floral organs. Most of the 942 genes that were underexpressed in the double mutant were expressed in multiple floral organs in other studies (Schmid et al., 2005; Wellmer et al., 2004), suggesting that ARF6 and ARF8 regulate a common set of genes in each organ. However, some of the genes were expressed specifically in particular floral organs, indicating that ARF6 and ARF8 also have organ-specific effects. For example, in the Schmid et al. (Schmid et al., 2005) dataset, 811 of the 942 genes were expressed in multiple floral organs, 59 were stamen specific and 13 were carpel specific. Further work may reveal how ARF6 and ARF8 interact with organ-specific factors to regulate different genes in different organs. The regulatory targets of ARF6 and ARF8 that are most relevant to its function in reproduction may be expressed in stamens and carpels.

JA feeding studies and JA measurements indicate that ARF6 and ARF8 regulate anther dehiscence by inducing JA production (or decreasing JA conjugation or breakdown). Several genes encoding JA biosynthetic enzymes are underexpressed in the double mutant, suggesting that gene expression changes underlie decreased JA production, although the positive feedback of JA synthesis means that the gene expression and feeding studies could not reveal the primary step at which ARF6 and ARF8 regulate JA level. JA in turn presumably regulates downstream genes required for anthesis. JA is thought to be produced in stamen filaments (Ishiguro et al., 2001), and it is therefore possible that *ARF6* and *ARF8* expression in filaments stimulates anther dehiscence.

JA did not rescue other *arf6-2 arf8-3* mutant phenotypes, and JA-deficient mutants are primarily deficient in anthesis. Therefore, ARF6 and ARF8 must activate other downstream effectors that regulate inflorescence stem elongation, bud

opening, filament elongation and carpel maturation. Some genes that depend on ARF6 and ARF8 encode putative regulatory proteins such as transcription factors (Table S4 in the supplementary material), and these may mediate secondary responses to ARF6 and ARF8.

### Auxin and flower development

Auxin might activate ARF6 and ARF8 activities by promoting turnover of Aux/IAA proteins that can inhibit ARF gene induction activity (Gray et al., 2001; Tian et al., 2003; Tian et al., 2002; Tiwari et al., 2003; Tiwari et al., 2001; Zenser et al., 2001). Consistent with this possible mechanism, some auxin-inducible genes were underexpressed in sesquimutant and double mutant flowers. However, wild-type flower bud auxin levels did not increase at stages 11-12 or 13-14, suggesting that a gross increase in auxin level does not induce flower maturation under our conditions. In fact, auxin is believed to be present in flower primordia from an early stage and to promote flower bud outgrowth (Benkova et al., 2003; Reinhardt et al., 2003). Auxin levels might change more locally, for example, within stamen filaments or ovules, or auxin may only be limiting under certain growth conditions. Increased auxin levels have been observed in flowers of a composite species at the stage of stamen filament elongation (Koning, 1983).

Mutations in the *ETTIN/ARF3* and *MP/ARF5* genes, encoding two other ARF proteins, affect flower organ numbers, and *ettin* mutant flowers also have expanded stigma and style (Przemeck et al., 1996; Sessions et al., 1997). These patterning defects occur at an earlier stage than the *arf6-2 arf8-3* flower arrest at stage 12, and *arf6-2 arf8-3* flowers had normal organ numbers and gynoecium patterning. Different auxin response factors thus regulate patterning and flower maturation, two very distinct aspects of flower development. These results imply that different ARF proteins may have some specificity in the promoters they bind or their interacting partners (Weijers et al., 2005).

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/18/4107/DC1>

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