

AUXIN RESPONSE FACTOR1* and *AUXIN RESPONSE FACTOR2* regulate senescence and floral organ abscission in *Arabidopsis thaliana

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Summary

In plants, both endogenous mechanisms and environmental signals regulate developmental transitions such as seed germination, induction of flowering, leaf senescence and shedding of senescent organs. Auxin response factors (ARFs) are transcription factors that mediate responses to the plant hormone auxin. We have examined *Arabidopsis* lines carrying T-DNA insertions in *AUXIN RESPONSE FACTOR1* (*ARF1*) and *ARF2* genes. We found that *ARF2* promotes transitions between multiple stages of *Arabidopsis* development. *arf2* mutant plants exhibited delays in several processes related to plant aging, including initiation of flowering, rosette leaf senescence, floral organ abscission and silique ripening. *ARF2* expression was induced in senescing leaves. *ARF2* regulated leaf senescence and floral organ abscission independently of the

ethylene and cytokinin response pathways. *arf1* mutations enhanced many *arf2* phenotypes, indicating that *ARF1* acts in a partially redundant manner with *ARF2*. However, unlike *arf2* mutations, an *arf1* mutation increased transcription of Aux/IAA genes in *Arabidopsis* flowers, supporting previous biochemical studies that indicated that *ARF1* is a transcriptional repressor. Two other ARF genes, *NPH4/ARF7* and *ARF19*, were also induced by senescence, and mutations in these genes enhanced *arf2* phenotypes. *NPH4/ARF7* and *ARF19* function as transcriptional activators, suggesting that auxin may control senescence in part by activating gene expression.

Key words: Auxin, Auxin response factor, Senescence, Abscission

Introduction

Plants progress through several distinct developmental phases during their life cycle, including seed dormancy, vegetative growth, flowering and senescence. In addition to environmental factors such as light, endogenous cues guide these transitions. Endogenous signals may arise from programmed events responding to age, as in vegetative phase change or the constitutive pathway for flower induction, or from metabolic status.

When plant structures have served their purpose, they senesce and are shed as part of this developmentally programmed sequence of events. Senescence enables the plant to marshal its resources to maximize its growth and reproductive capacity, and contributes to pathogen defense and environmental stress responses. Plant hormones that influence senescence include ethylene, abscisic acid and jasmonates, which can induce senescence; and auxin, cytokinin and gibberellins, which can play a role in its suppression (Lim et al., 2003). Auxin represses transcription of some genes whose expression is correlated with senescence and/or abscission (Noh and Amasino, 1999; Hong et al., 2000; Tucker et al., 2002).

The auxin response factor (ARF) family of transcription

factors regulate many responses to auxin. These proteins bind to auxin response elements (5'-TGTCTC-3') in the promoters of auxin regulated genes and either activate or repress transcription of these genes (Ulmasov et al., 1997a). *Arabidopsis* has 22 genes encoding ARF proteins (Remington et al., 2004). Most ARFs have three domains: an N-terminal DNA-binding domain, a C-terminal dimerization domain that is similar to domains III and IV of the Aux/IAA family of proteins, and a middle region (MR) that activates or represses transcription (Ulmasov et al., 1999a; Ulmasov et al., 1999b). ARFs containing glutamine-rich MRs function as activators of auxin responsive gene expression in transiently transfected protoplasts (Ulmasov et al., 1999a) and in vivo (Wilmoth et al., 2005; Okushima et al., 2005a; Nagpal et al., 2005). These include MP/ARF5, which is involved in embryo patterning and vascular formation (Hardtke and Berleth, 1998), *NPH4/ARF7*, which is involved in phototropism and gravitropism (Harper et al., 2000), *ARF19*, which acts redundantly with *NPH4/ARF7* in controlling leaf expansion and lateral root growth (Okushima et al., 2005a; Wilmoth et al., 2005), and *ARF6* and *ARF8*, which act redundantly in flower maturation (Nagpal et al., 2005). The activity of these ARFs is negatively regulated

by heterodimerization with Aux/IAA proteins (Reed, 2001; Tiwari et al., 2003).

ARFs containing proline- and/or serine-rich MRs repress auxin responsive gene expression in protoplast transient assays (Tiwari et al., 2003). These include the closely related proteins ARF1 and ARF2. Mutations in *ARF2* partially restore apical hook formation to dark-grown *hookless1* (*hls1*) seedlings, and ethylene promotes ARF2 protein turnover in etiolated seedlings in a HLS1-dependent manner (Li et al., 2004). Dark-grown *arf1 arf2* double mutant seedlings had an exaggerated hypocotyl hook, whereas single mutants resembled wild-type plants, indicating redundancy between these two genes. These results led to the model that ARF1 and ARF2 act downstream of *HLS1*, and that they integrate ethylene and light signals to control apical hook formation. *arf2* mutations restored asymmetric *DR5::GUS* expression in apical hooks of dark-grown *hls1* seedlings (Li et al., 2004), suggesting that ARF2 may repress auxin-regulated gene expression. Alternatively the restoration of asymmetric *DR5::GUS* expression may have been an indirect effect of restoration of the apical hook. Other workers have found that *arf2* mutant plants have enlarged seeds, stems and cotyledons, and elongated hypocotyls under red light, but did not find significant effects on auxin-responsive gene expression (Schruff et al., 2005; Okushima et al., 2005b).

In this report, we show that *ARF2* and *ARF1* promote transitions between developmental phases. Lines carrying T-DNA insertions in these genes were isolated and lines with decreased expression of *ARF2* were also created using double-stranded RNA interference (dsRNAi). The plants with decreased *ARF2* flowered late, and had delayed senescence of rosette leaves and delayed abscission of floral organs. In addition ARF1 acted as a repressor of auxin-induced genes. *arf1* and *arf2* mutations had synergistic effects on some phenotypes and independent effects on others, indicating both redundancy and specialization of ARF1 and ARF2 function. Furthermore, *nph4/arf7* and *arf19* mutations also enhanced the *arf2* senescence and abscission phenotypes, suggesting that ARFs of different functional classes can regulate common processes.

Materials and methods

Isolation of alleles and creation of RNAi lines

We isolated plants with mutations in *ARF1* or *ARF2* in Wassilewskija (Ws-0) and Columbia wild-type backgrounds. To isolate the *arf1-4* insertion in Ws-0, we designed a degenerate oligodeoxyribonucleotide (ARFdIV-1, 5'-CCATGGATCATCW-CCWACMABCATCATRTC-3') predicted to hybridize to DNA-encoding conserved domain IV of multiple members of the *Arabidopsis* ARF gene family. We amplified from template DNA derived from pools of T-DNA insertion lines, using ARFdIV-1 and primers specific to the ends of the T-DNA as described previously (Young et al., 2001). The *arf1-5* line (SALK_079046) and two *arf2* lines (*arf2-5*, SALK_041472 and *arf2-8*, SALK_108995) in Columbia were isolated from the SALK collection (Alonso et al., 2003; Li et al., 2004). PCR products amplified using T-DNA border primers and gene-specific primers were sequenced to verify the sites of the insertions. Both *arf2* lines exhibited similar phenotypes and the *arf2-8* line was characterized in greater detail. In addition, a dsRNAi construct was created by cloning the region that corresponded to nucleotides 1307 to 1743 of the coding region of *ARF2* (Fig. 1) into the pB7GWIWG2(II) vector (Karimi et al., 2002). This was

transformed into Columbia, Ws-0, and *arf1-4* plants using the floral dip method (Clough and Bent, 1998). Transgenic lines were selected for resistance to 0.04% phosphinothricin herbicide ('Liberty', Bayer CropScience). One *arf1-4 dsARF2* line that segregated 3:1 for antibiotic resistance and exhibited decreased fertility and delayed floral organ abscission was chosen for detailed analysis. This line was crossed once to wild-type plants to generate a Ws-0 (*ARF1 dsARF2*) line. F2 individuals that were homozygous for the wild-type *ARF1* allele were isolated and F3 plants homozygous for the RNAi transgene were studied further.

Analysis of ARF and AUX/IAA gene expression

P_{ARF1}::GUS reporter lines were created by digesting DNA from BAC AC007258 with restriction endonucleases *XbaI* and *SacI*. A 2.6 kb fragment encompassing the ARF1 promoter and start codon was cloned into pBluescript SK+ (Promega). This plasmid was used as a template to amplify a 2.2 kb fragment containing the ARF1 5' upstream regions using the T7 primer and the gene specific primer 5'-GGAAGCTGCCATGGGGAATC-3'. This fragment was cloned into the *SalI/NcoI* sites of the pEBGUS vector (Hagen et al., 1991). The ARF1 promoter-GUS-nos fragment was excised with *EcoRI* and cloned into pPZP211. *P_{ARF2}::GUS* lines were created by amplifying a 2.5 kb fragment from BAC AB016880 DNA using the primers: 5'-CACACAAATGCTGCAGAGTACTCTTGGGTC-3' and 5'-CGC-AGGATCCGAAGCTCAGATCTGTTTCATCTGG-3'. Seven plant lines per construct were analyzed for GUS activity and representative lines were chosen for further analysis. *P_{IAA3}::GUS* and *P_{IAA7}::GUS* lines were described previously (Tian et al., 2002). Plant tissue was stained for GUS activity as described previously (Wilmoth et al., 2005).

Total RNA was isolated from seedlings grown in liquid medium as described previously (Tian et al., 2002) or from leaves or flowers of adult plants using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Poly (A⁺) RNA was extracted using oligo(dT)₂₅ Dynabeads according to manufacturers' instructions (Dyna, Lake Success, NY). RNA gel blot hybridizations were performed as described (Nagpal et al., 2000; Tian et al., 2002) using 30 µg of total RNA or mRNA derived from 50 µg of total RNA. Probes were created by PCR using genomic DNA or cDNA as template and the following primers: *ARF1*, 5'-CCTAATGGCAGCTTCCAATCAT-3' and 5'-GCAAATGACGCCTTGGTTGGC-3'; *ARF2*, 5'-GAAGGTATGGC-GAGTTCCGGAGG-3' and 5'-GGAGGCTGTGAGACATATC-3'; *IAA3*, 5'-CTTCATCATCAGCAGCTTCTCT-3' and 5'-GGGGAT-GTTTCAGTTTTTCTT-3'; *IAA7*, 5'-CAAGTAACATGATCGGC-CAAC-3' and 5'-GGCTTAGAAGGATCTTTAAGGGGTA-3'; *SAG12*, 5'-GATGTTGTTGGCGTTTTTCAGCGG-3' and 5'-CTTTCATGGCAAGCCACATAGTCC-3'; *IAA2*, 5'-CTTAT-GATCCAGAGAAGCTGAGAATC-3' and 5'-ATCTCATGTATCTT-TACATCAAACCTTC-3'; and *IAA5*, 5'-TTCCCATGAGAACATATA-AAGTGG-3' and 5'-TGACTCTTTTTTCGCCGGTTC-3'; *PDF1.2*, 5'-ATGGCTAAGTTTGCTTCCATCATC-3' and 5'-ACATGGGA-CGTAACAGATACACTT; *ERF1*, 5'-CCACTTCAAACCTTAA-GGTCCCTA-3' and 5'-ATGGATCCATTTTTAATTCAGTCC-3'; *ARF7*, 5'-TCCTGCTGAGTTTGTGGTTCCTT-3' and 5'-GGGG-CCTTGCTGATTCTGTTTGTGA-3'; and *ARF19* 5'-ACGATTGCT-GTTGCTAACCA-3' and 5'-GGAATGCTGGGAATACAAA-3'.

Determination of chlorophyll levels

For senescence assays, plants were grown under short day conditions for 6 weeks, at which time the plants had approximately 18 leaves visible to the naked eye. The 9th and 10th leaves were excised and either frozen in liquid nitrogen for RNA extraction or placed in a microfuge tube containing 50 µl of water so that the petiole was immersed in water. The microfuge tubes were then placed in the dark for various lengths of time. Chlorophyll content was determined spectrophotometrically and normalized to fresh weight as described previously (Porra et al., 1989).

Histology

Samples were fixed overnight in 4% glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.0, then washed twice with buffer. Samples were dehydrated in a graded ethanol series to 100% ethanol, then embedded in London Resin White medium grade resin (Sigma) according to manufacturer's instructions. Sections (1 μ m) were obtained using a Sorvall Porter-Blum MT2-B ultramicrotome, heat-fixed to a glass slide, then stained with 0.5% Toluidine Blue.

Results

Isolation of plants deficient in *ARF1* and *ARF2*

To study functions of *ARF1* and *ARF2*, we isolated T-DNA insertions in them (Fig. 1A, Materials and methods). The *arf1-4* insertion lies within the middle region, which is required for transcriptional repression (Ulmasov et al., 1999a), while the *arf1-5* insertion is located within domain IV, which, along with domain III, is responsible for dimerization. The *ARF1* transcript was not detected by RNA gel blot hybridization in *arf1-4* plants in the Ws-0 ecotype. *arf1-5* plants had a near full-length transcript present at lower level than in wild-type Columbia plants (Fig. 1B). Presumably this transcript had T-DNA insertion sequences at the 3' end instead of sequences encoding the normal *ARF1* C terminus, and this allele may therefore produce a decreased level of *ARF1* with a slightly altered C terminus, which may retain some *ARF1* function.

ARF2 mRNA was not detected in the *arf2-8* line (Fig. 1B), which contains an insertion in the middle region. We also made a dsRNAi construct to silence the *ARF2* gene (Fig. 1A) and transformed this construct into Columbia, Ws-0 and *arf1-4* backgrounds. In the Columbia background, 32 out of 44 T1 plants exhibited decreased fertility and delayed floral organ abscission similar to *arf2-8* (see below). In the Ws-0 and *arf1-4* backgrounds, two out of eight and 37 out of 58 T1 plants, respectively, displayed wild-type fertility but exhibited delays in floral organ abscission. Five *arf1-4 dsARF2* T1 plants exhibited decreased fertility, similar to *arf2-8* plants. These plants also had delayed floral organ abscission. The transgene from a strong *arf1-4 dsARF2* line that segregated 3:1 for antibiotic resistance was crossed into the Ws-0 background (see Materials and methods) and this line was further characterized in this study. A reduced amount of *ARF2* transcript was observed in this dsRNAi line (Fig. 1B), indicating that these plants may retain some *ARF2* function.

Mutations in *ARF1* and *ARF2* delay rosette leaf senescence

arf2 plants exhibited delays in the natural senescence of rosette leaves. After 8 weeks of growth under long day conditions, the oldest leaves of Columbia plants typically had senesced and withered, whereas those from *arf2-8* plants remained green and turgid (Fig. 2). The *arf2-8* plants also produced more flowers than did Columbia plants, presumably because of prolonged growth. In one experiment, Columbia plants produced 60 ± 4 flowers ($n=10$) on the primary inflorescence when compared with 74 ± 5 ($n=14$) for *arf2-8* plants. In addition, the majority of Columbia flowers had set seed, while *arf2* plants had reduced fertility (see below). However, although some sterile mutants have delayed senescence (Robinson-Beers et al., 1992), reduced fertility is unlikely to have caused the delay in senescence because, in *Arabidopsis*, unlike some other monocarpic species, plant age rather than successful seed set induces rosette leaf senescence, and sterility is not sufficient to alter the timing of leaf senescence (Hensel et al., 1993).

We also used a detached leaf assay to study the effects of *arf1* and *arf2* mutations on dark-induced senescence in plants prior to flowering. Although some differences in gene expression patterns exist between natural and dark-induced senescence (Becker and Apel, 1993; Weaver et al., 1998), detached leaf assays have been used as a convenient method of accelerating leaf senescence (Oh et al., 1997; Lin and Wu, 2004). Rosette leaves from 6-week-old plants that had been grown under short day conditions were placed in darkness for 8 days prior to chlorophyll measurement. Under these conditions, *arf1-4* and *arf1-5* leaves lost a similar amount of chlorophyll, as did leaves of wild-type plants, whereas *arf2-8*, *Ws-0 dsARF2*, *arf1-5 arf2-8* and *arf1-4 dsARF2* plants retained more chlorophyll than did wild-type plants (Fig. 3A). Most of the chlorophyll retention was attributed to the loss of *ARF2* and a time course was conducted to compare Columbia and *arf2-8* plants. Columbia leaves lost chlorophyll at a faster rate than did *arf2-8* leaves, starting after 2 days in darkness (Fig. 3B).

To determine whether other aspects of leaf senescence were affected, RNA gel blots were used to examine the expression of *SENESCENCE ASSOCIATED GENE 12* (*SAG12*), a marker for senescence (Lohman et al., 1994). Whereas many *SAG* genes respond to multiple senescence-inducing factors (He et al., 2001), *SAG12* is specifically activated by developmental regulation and not by hormone- or stress-controlled pathways

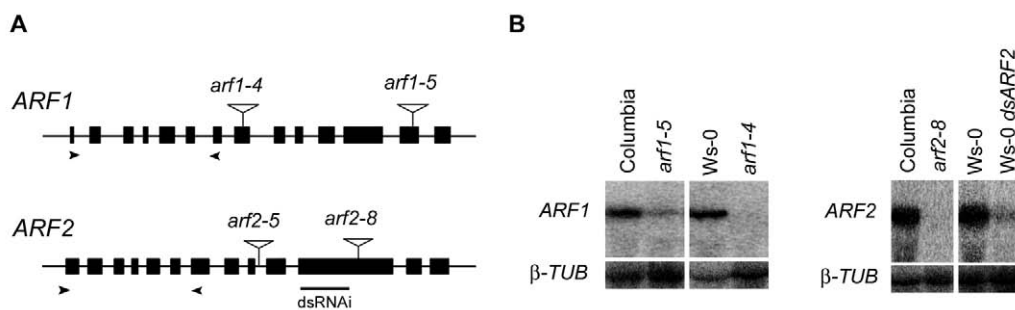


Fig. 1. Identification of *ARF1* and *ARF2* T-DNA insertions. (A) Positions of T-DNA insertions in *ARF1* and *ARF2*. Boxes represent exons, lines represent non-coding regions, and inverted triangles indicate T-DNA insertions. The region used for creation of a dsRNAi construct is indicated by a bar. Arrowheads indicate the locations of primers used to create probes. (B) Northern blot analysis of *ARF* gene expression in T-DNA insertion mutants and *dsARF2* plants. RNA was extracted from 10-day-old seedlings.



Fig. 2. Natural senescence in wild-type and *arf2* rosette leaves. Leaves and inflorescences from 8-week-old plants are laid out in order of emergence.

(Weaver et al., 1998) and is therefore an excellent marker gene for development-mediated senescence. Leaves from Columbia plants that had been placed in the dark accumulated *SAG12* mRNA starting after 2 days, and *SAG12* levels peaked after 4 days (Fig. 3C). *arf2-8* leaves accumulated very little *SAG12* even after 6 days (Fig. 3C). These data show that ARF2 is required for normal leaf senescence both in intact plants and in detached leaves. By contrast, *SAG12* expression levels and chlorophyll loss were similar in *Ws-0* and *arf1-4* plants, suggesting that ARF1 on its own has little effect on senescence.

In wild-type plants, levels of ARF2 mRNA also increased in detached leaves following dark treatment, with a time course similar to that of *SAG12* mRNA (Fig. 3C). Microarray studies also indicated that ARF2 mRNA increased during senescence (Lin and Wu, 2004). We examined *P_{ARF2}::GUS::ARF2* plants (Li et al., 2004) to determine the levels of ARF2 protein in senescing leaves. In mature, fresh fully expanded rosette leaves or in those placed in the dark for 2 days, little GUS staining was observed (Fig. 4A,B). After 4 and 6 days in darkness, GUS staining was evident (Fig. 4C,D). GUS activity was highest in vasculature, but was also present in ground tissue. Together these data indicate that ARF2 accumulated during leaf senescence.

Younger, expanding leaves of *P_{ARF2}::GUS::ARF2* plants also stained in the vasculature and ground tissue, both in freshly detached leaves and in those placed in the dark for 4 days (Fig. 4E,F), suggesting that ARF2 may also function early in leaf development during leaf expansion.

In contrast to ARF2, ARF1 mRNA decreased as senescence progressed in both Columbia and *arf2-8* plants (Fig. 3C). ARF1 mRNA levels were quantified in three independent blots and were found to be slightly higher (2.7 ± 0.9 -fold) in freshly detached *arf2* leaves when compared with Columbia leaves. These patterns suggest that ARF1 has a different function than does ARF2 during wild-type leaf senescence.

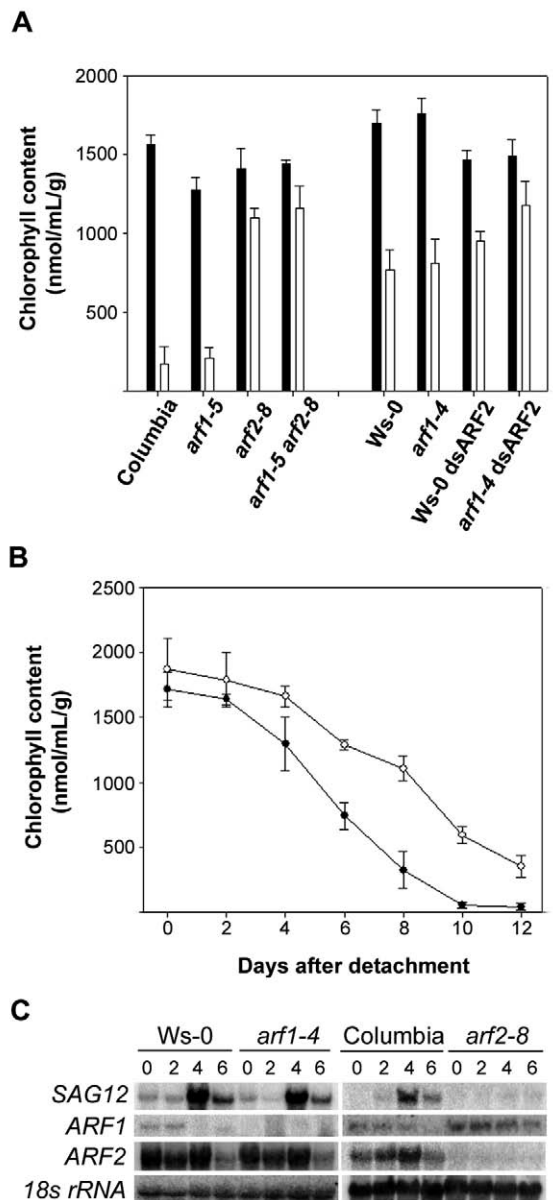


Fig. 3. Dark-induced senescence in detached wild-type and mutant leaves. (A) Chlorophyll levels in detached leaves following an 8 day incubation in the dark. Black bars represent chlorophyll levels just after detachment and white bars represent chlorophyll levels of leaves incubated in the dark for 8 days. Error bars indicate s.d. ($n=6$). (B) Time course of chlorophyll loss in Columbia and *arf2-8* plants. Black circles indicate chlorophyll levels of Columbia leaves and white circles indicate *arf2-8* leaves. (C) Northern blot analysis of gene expression in detached leaves. Leaves from Columbia and *arf2-8* plants were detached, placed in darkness for various time periods, and then used for chlorophyll measurement or RNA extraction. Error bars indicate s.d. ($n=6$).

ARF1 and ARF2 promote flowering, stamen development, floral organ abscission, and fruit dehiscence

Flowering time was affected by mutations in ARF1 and ARF2. Plants were grown under long day conditions until the inflorescence was ~1 cm in height. At this stage, the *arf1-4* and

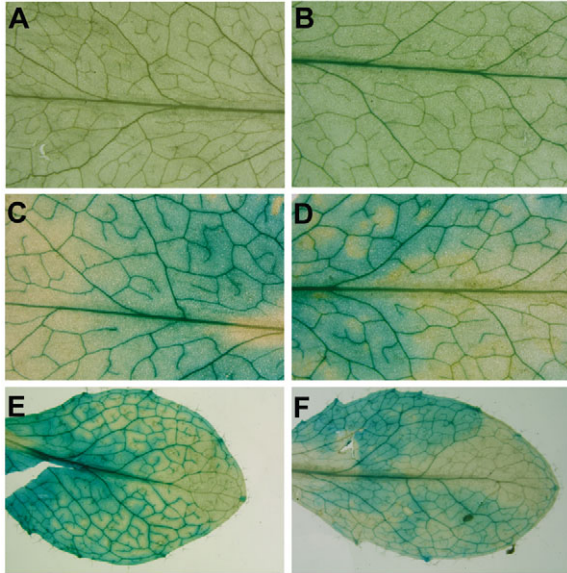


Fig. 4. GUS::ARF2 protein levels during dark-induced senescence. *P_{ARF2}::GUS::ARF2* plants (Li et al., 2004) were grown under short day conditions for 6 weeks. Mature, fully expanded leaves (A-D) or young, expanding leaves (E,F) were detached and stained immediately (A,E) or were placed in darkness for 2 days (B), 4 days (C,F) or 6 days (D) prior to staining.

arf1-5 lines did not differ statistically from corresponding wild-type plants either in leaf number or in plant age. However, the transition to flowering in *arf2-8* and *Ws-0 dsARF2* plants was delayed by ~3 leaves and 5 days (Table 1). Furthermore, the double mutants *arf1-4 dsARF2* and *arf1-5 arf2-8* were also delayed by ~4 leaves and 6 days.

Although *arf1-4* and *arf1-5* plants were completely fertile, both *Ws-0 dsARF2* and *arf2-8* plants exhibited reduced fertility. In wild-type *Arabidopsis*, anthers normally dehisce when flowers open at stage 13 (Smyth et al., 1990). At this time, the stamens and carpels are of approximately equal height (Table 1, Fig. 5A) so that pollen can be deposited easily onto the stigmatic papillae and fertilization can occur. In *arf2-8* flowers at anthesis, stamens were shorter than wild-type stamens, and carpels were slightly longer, so stamen height was only 70% of carpel height (Table 1, Fig. 5E). *Ws-0 dsARF2* flowers had a slightly less severe defect, with stamen length at 88% of carpel length. *arf1-4 dsARF2* plants and *arf1-5 arf2-8* plants had an even more pronounced difference between stamen and carpel lengths, and were almost completely infertile (Table 1). These plants set seed when manually pollinated, either with their own or with wild-type pollen, suggesting that the cause of infertility was the failure of pollen to reach the stigma. Stamen lengths were normal at the 2 mm bud stage (Table 1), indicating that the defect occurs late in stamen development, probably at stage 12 when filaments elongate.

In *Arabidopsis*, shortly after flowers open and pollination occurs, fertilized siliques begin to expand, and sepals, petals and stamens wither and abscise (Smyth et al., 1990). We measured timing of organ abscission in two ways. The first method was to identify the position of the first flower whose perianth organs had been shed, where newly opened flowers

are designated as position 1 and flowers are numbered basipetally onwards (Patterson, 2001; Patterson and Bleeker, 2004). Wild-type and *arf1* single mutant plants typically shed perianth organs at approximately position 6.5, whereas *Ws-0 dsARF2* and *arf2-8* plants typically retained their perianth organs until about position 8 (Table 1). The *arf1-4 dsARF2* plants and *arf1-5 arf2-8* lines did not shed floral organs until positions 9.6 and 9.0, respectively. The second method of determining the timing of organ abscission was to measure the length of the silique at abscission. In *Ws-0* and Columbia, flowers shed their perianth organs when the silique was ~4.5 mm long (Table 1), with fully expanded siliques reaching a length of 11.3 ± 1.0 mm. *arf1-4* and *arf1-5* plants did not differ significantly from wild-type plants. However, in *arf2-8* and *dsARF2* plants, floral organ abscission was delayed until the siliques reached ~10.0 and 9.0 mm long, respectively. The delay of organ shedding was also more pronounced in the *arf1-5 arf2-8* plants and in *arf1-4 dsARF2* lines, which did not shed perianth organs until the silique was 10.6 and 10.2 mm, respectively (Fig. 5F,G). In addition to being shed later than wild-type sepals, sepals of *arf2* and *arf1 arf2* plants remained green and turgid until detachment, whereas wild-type sepals turned yellow prior to detachment (Fig. 5B,C).

In these experiments, *arf2* single and double mutant lines were manually pollinated to ensure that differences in perianth shedding were not due to changes in signals from fertilized siliques to outer whorl organs. However, manual pollination did not affect the position at which floral organs were shed (data not shown). Furthermore, whereas strong *arf1-4 dsARF2* lines were infertile, weaker lines set seed normally. These fertile lines also exhibited delays in floral organ abscission (Fig. 5G), confirming that delays in floral organ abscission did not correlate with sterility.

Cells destined to become the abscission zone can be identified prior to separation by their morphology. Abscission zone cells are located at the base of floral organs and are typically smaller and more densely cytoplasmic than neighboring non-separating cells (Roberts et al., 2002). To determine whether abscission zones formed normally in the mutant, flowers from position 4 (two flowers prior to floral organ abscission in wild-type plants) were sectioned from Columbia and *arf1-5 arf2-8* plants (Fig. 5D,H). In both plants, an abscission zone comprising a few layers of small cells was present near the base of the floral organs. Hence, at this level of resolution, the abscission zones looked similar in both mutant and Columbia plants. This suggests that the formation of the abscission zone proceeded normally in *arf2* plants, but the timing of cell separation was delayed. This result is consistent with those of Hanisch Ten Cate and Bruinsma (Hanisch Ten Cate and Bruinsma, 1973) who found that although IAA treatment delayed abscission in *Begonia* flower bud pedicels, it did not affect the anatomy of the abscission zone. Auxin inhibits the expression of some cell wall degrading enzymes (Hong et al., 2000; Tucker et al., 2002) and may therefore delay cell separation rather than abscission zone patterning.

Two to three weeks after pollination, *Arabidopsis* siliques dry and shatter, releasing the mature seed. ARF1 and ARF2 also influenced the timing of silique dehiscence. Dehiscence was delayed by approximately 4 days relative to pollination in *arf2* and *arf1 arf2* mutants (Table 1). We also observed that siliques of *arf2* plants remained green for longer than wild-type siliques.

Table 1. Measurements of wild-type and mutant plants

Genotype	Columbia	<i>arf1-5</i>	<i>arf2-8</i>	<i>arf1-5 arf2-8</i>	<i>Ws-0</i>	<i>arf1-4</i>	<i>Ws-0 dsARF2</i>	<i>arf1-4 dsARF2</i>
Number of rosette leaves before bolting	12.2±1.8 (12)	12.5±1.2 (11)	15.0±1.4 (9) [†]	16.2±1.0 (7)	9.2±0.6 (10)	7.8±0.6 (10)	11.1±1.3 (10) [†]	13.6±1.6 (7) [‡]
Time to bolting (days)	26.2±0.9 (12)	26.7±1.8 (11)	31.1±0.9 (9) [†]	31.3±1.3 (7)	22.7±0.5 (10)	23.1±0.6 (10)	25.8±1.6 (10) [†]	29.1±1.7 (7) [‡]
Stamen length of 2 mm bud (mm)	1.3±0.1 (20)	1.3±0.1 (20)	1.3±0.1 (20)	1.4±0.1 (20)	1.3±0.1 (20)	1.3±0.1 (20)	1.3±0.1 (20)	1.2±0.1 (20)
Stamen length at anthesis	2.1±0.1 (25)	2.2±0.2 (25)	1.7±0.2 (25) [†]	1.5±0.3 (25) [‡]	2.5±0.2 (25)	2.5±0.1 (25)	2.2±0.2 (25) [†]	1.6±0.2 (25) [‡]
Carpel length at anthesis	2.3±0.2 (25)	2.3±0.2 (25)	2.5±0.2 (25)	2.4±0.2 (25)	2.4±0.2 (25)	2.4±0.3 (25)	2.5±0.2 (25)	2.4±0.2 (25)
Average position at abscission*	6.5±1.1 (32)	5.9±0.8 (30)	8.0±0.7 (29) [†]	9.0±0.8 (25) [†]	6.1±0.9 (34)	6.5±0.8 (32)	7.9±1.2 (30) [†]	9.6±1.3 (41) [‡]
Silique length at floral organ abscission (mm)	4.5±0.7 (136)	4.5±0.8 (118)	10.0±1.8 (55) [†]	10.6±1.9 (65)	4.6±0.7 (111)	4.5±0.6 (109)	9.0±1.4 (60) [†]	10.2±2.1 (94) [‡]
Time to silique dehiscence (DAP)	19.3±1.2 (32)	19.2±2.4 (29)	24.0±1.3 (28) [†]	23.4±2.2 (31)	18.9±1.3 (28)	19.5±1.5 (27)	20.4±1.8 (24) [†]	23.6±2.2 (28) [‡]

Values given are mean±s.d. (n)
 *Average position at abscission where position 1 equals the first opened (stage 13) flower and flowers are number onwards basipetally.
[†]Indicates that *arf1*, *arf2* or *dsARF2* single mutant values are distinguishable from corresponding wild-type values by *t*-test ($P<0.05$).
[‡]Indicates that *arf1-5 arf2-8* or *arf1-4 dsARF2* values are distinguishable from corresponding *arf2-8* or *dsARF2* values by *t*-test ($P<0.05$).
 DAP, days after pollination.

In summary, *arf2* mutations had a major effect on senescence of rosette leaves, stamen length, floral organ abscission, flowering time and silique dehiscence. Mutations in the *ARF1* gene typically enhanced the effect of *arf2* mutations. This was evident in both the *arf1-4* and *arf1-5* alleles for stamen elongation and the timing of floral organ abscission. However, only the stronger, transcript null *arf1-4* allele also enhanced the delayed leaf senescence and flowering time phenotypes.

Expression of *ARF1* and *ARF2*

ARF1 and *ARF2* mRNA are each present in roots, rosette leaves, cauline leaves and flowers (Ulmasov et al., 1999b). To examine the expression domains of *ARF1* and *ARF2* further, we made transgenic plant lines carrying 2.2 kb of the *ARF1* promoter or 2.5 kb of the *ARF2* promoter upstream of the *GUS* gene and examined *GUS* expression patterns by X-Gluc

staining. In both *P_{ARF1}::GUS* and *P_{ARF2}::GUS* lines, X-Gluc staining appeared throughout 8-day-old seedlings and in rosette leaves (data not shown). In addition, stain appeared in the sepals and carpels of young flower buds of both *P_{ARF1}::GUS* and *P_{ARF2}::GUS* lines (Fig. 6A,D). Staining in the carpels became restricted to the style at approximately stage 10, at which time staining also appeared in anthers and filaments. Sepal, stamen and carpel staining persisted until floral organs were shed (Fig. 6B,E). From stage 13, *GUS* activity also appeared in the region at the top of the pedicel, including the abscission zone (Fig. 6C,F). *P_{ARF2}::GUS::ARF2* plants (Li et al., 2004) showed similar staining patterns as *P_{ARF2}::GUS* plants (data not shown), suggesting that the *P_{ARF2}::GUS* patterns observed reflect the true expression pattern of *ARF2*. Furthermore, the *GUS* activity was consistent with microarray expression profiles that found *ARF1* and *ARF2* mRNA in all four floral whorls (Schmid et al., 2005).

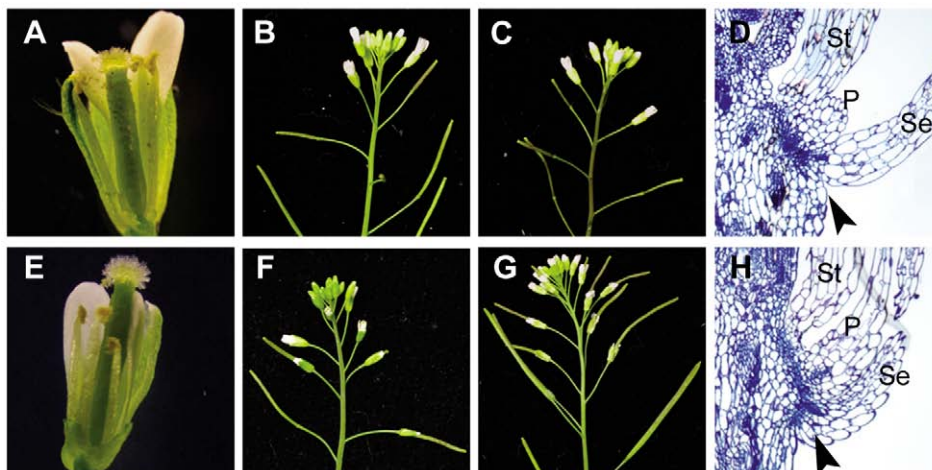


Fig. 5. Effects of *arf* gene mutations on flower development. Stage 13 Columbia (A) and *arf2-8* flowers (E). Primary inflorescences of Columbia (B), *Ws-0* (C) *arf1-5 arf2-8* (F) and *arf1-4 dsARF2* (G) plants. Abscission zones of Columbia (D) and *arf1-5 arf2-8* (H) floral organs. St, stamen; P, petal; Se, sepal. Arrowheads indicate the abscission zone.

The *arf1-4* mutation, but not the *arf2-8* mutation, affects IAA gene expression in the floral abscission zone

In transient assays, both ARF1 and ARF2 can repress expression from promoters containing AuxRE sequences (Tiwari et al., 2003; Ulmasov et al., 1999b). To determine whether *arf1* and *arf2* mutations derepress such genes in planta, we examined the expression of endogenous IAA genes whose upstream regions are rich in AuxREs, and of promoter::GUS fusions to *IAA3* and *IAA7* genes (Tian et al., 2002). In wild-type plants, *P_{IAA3}::GUS* was expressed in hypocotyls, cotyledons and expanding leaves, and *P_{IAA7}::GUS*

was expressed in root and shoot meristems (Tian et al., 2002), but neither was detectable in flowers (Fig. 6G,H,M,N). In the *arf1-4* mutant, both fusions were overexpressed (Fig. 6I,J,O,P). *P_{IAA3}::GUS* and *P_{IAA7}::GUS* X-Gluc staining was present in the anthers and in the floral abscission zone beginning at stage 13 (Fig. 6I,O), and *P_{IAA3}::GUS* activity was present in sepals of opened flowers (Fig. 6I). Ectopic *P_{IAA3}::GUS* and *P_{IAA7}::GUS* stain was also observed in the leaves of *arf1-4* seedlings (data not shown).

RNA gel blot hybridizations confirmed that endogenous *IAA3* and *IAA7* mRNA levels were increased in *arf1-4* flowers and seedlings, and in *arf1-4 arf2-8* flowers (Fig. 6S). Furthermore, *IAA2* mRNA level was increased in *arf1-4* and *arf1-4 arf2-8* flowers, and both *IAA2* and *IAA5* mRNA levels were increased in auxin-treated *arf1-4* seedlings. RNA levels of *IAA2*, *IAA3*, *IAA5* or *IAA7* transcripts in *arf2* flowers (Fig. 6S) and seedlings (data not shown) were similar to those of Columbia plants.

Whereas *arf1-4* plants had strongly derepressed *P_{IAA3}::GUS* and *P_{IAA7}::GUS* activity, increased GUS activity was detected in some but not all *arf1-5* plants carrying *P_{IAA3}::GUS* or *P_{IAA7}::GUS* fusions. This may reflect differences in the nature of the insertions, as the *arf1-4* allele is a transcript null mutant and probably stronger than the *arf1-5* allele, which produces some *ARF1* transcript. Alternatively, the difference between the two alleles may be due to differential effects between the *Ws-0* and Columbia ecotypes.

Although *arf2* mutants had stronger organ abscission phenotypes than *arf1* mutants, no stain was visible in the floral abscission zones of *arf2-6* or *Ws-0 dsARF2* plants carrying *P_{IAA3}::GUS* or *P_{IAA7}::GUS* fusions (data not shown). *arf1-4 arf2-8* lines stained in the same locations as did *arf1-4* lines (Fig. 6K,L,Q,R). It is possible that ARF2 also represses IAA gene expression, but the upregulation of *ARF1* in the *arf2* background (Fig. 3C) may counteract the loss of ARF2 and result in the overall lack of ectopic expression of *P_{IAA3}::GUS* and *P_{IAA7}::GUS* in *arf2-8* plants. However, *P_{IAA3}::GUS* and *P_{IAA7}::GUS* stain appeared similar in both the *arf1-4* and *arf1-4 arf2-8* flowers, and they had similar levels of *IAA3* and *IAA7* mRNA, indicating that ARF2 had no further effect on the expression of these genes.

Taken together, these results indicate that ARF1 and ARF2 have distinct effects on gene expression in flowers. ARF1 represses expression of some auxin-inducible genes, consistent with its ability to repress gene expression in protoplast assays (Tiwari et al., 2003; Ulmasov et al., 1999b). However, mutation of *ARF2* did not affect expression of these genes.

ARF2 functions independently of ethylene and cytokinin response pathways

Ethylene insensitive plants such as *etr1* and *ein2* have some characteristics in common with *arf2* plants, such as delayed floral organ abscission and delayed leaf senescence (Bleecker and Patterson, 1997). We therefore made *arf2-8 ein2-1* double mutant lines to determine how *ARF2* might interact with the ethylene signaling pathway to promote senescence. Both *ein2* and *arf2* single mutant leaves had reduced dark-induced chlorophyll loss. After incubation in the dark for 8 days, Columbia leaves retained only 18% of their chlorophyll, while *arf2-8* and *ein2-1* leaves retained 45% and 49%, respectively (Fig. 7A). *arf2-8 ein2-1* leaves retained 76% of their

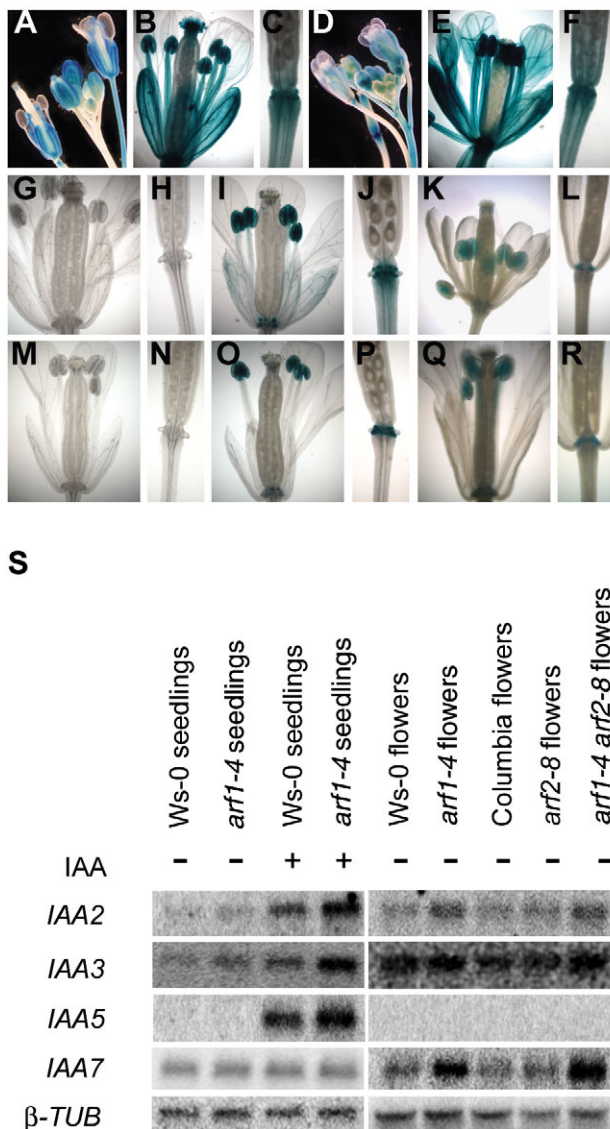


Fig. 6. Analysis of *ARF* and *IAA* promoter activity in *Arabidopsis* flowers. *P_{ARF1}::GUS* (A-C) and *P_{ARF2}::GUS* (D-F) activity in flower bunches (A,D), stage 13 flowers (B,E) and stage 17 flowers (C,F). *P_{IAA3}::GUS* (G-L) and *P_{IAA7}::GUS* (M-R) activity in stage 13 flowers (G,I,K,M,O,P) and stage 17 flowers (H,J,L,N,P,R) in wild-type (G,H,M,N), *arf1-4* (I,J,O,P) and *arf1-4 arf2-8* (K,L,Q,R) plants. (S) Northern blot analysis of *IAA* gene mRNA in seedlings and flowers. Seedlings were treated for 2 hours with 1 μ M IAA or with a mock control.

chlorophyll, indicating that the mutations act additively and that delayed senescence in *arf2* plants cannot be attributed solely to alterations in ethylene signaling.

arf2 and *ein2* mutations also had an additive effect on the number of opened flowers that retained their floral organs. Whereas Columbia flowers shed their perianth at position 5.3, *arf2-8* and *ein2-1* flowers shed their perianth at positions 8.8 and 7.3, respectively, and flowers from *arf2-8 ein2-1* shed their outer whorl organs at position 10.2 (Fig. 7B).

Ethylene induces expression of *PDF1.2* and *ERF1* in rosette leaves (Penninckx et al., 1998; Solano et al., 2000). Exogenous ethylene induced these genes to a similar level in both Columbia and *arf2-8* leaves, suggesting that *arf2-8* plants have normal sensitivity to exogenous ethylene (Fig. 7C). Furthermore, detached *arf2-8* rosette leaves produced similar amounts of ethylene as Columbia leaves (data not shown). These results indicate that ARF2 controls senescence and floral organ abscission independently of ethylene.

P_{SAG12}::IPT plants that produce a burst of cytokinin in response to the onset of senescence show delays in leaf senescence (Gan and Amasino, 1995) and disruption of the cytokinin signaling pathway affects the rate of dark-induced chlorophyll loss (To et al., 2004). Auxin and cytokinin are known to interact on many levels (Coenen and Lomax, 1997) and it is therefore possible that the effect of ARF2 on senescence is mediated by cytokinin. To test this hypothesis, detached leaves from Columbia and *arf2-8* plants were treated with cytokinin to determine whether the mutant plants respond differentially to hormone treatment (Fig. 7D). Cytokinin delayed chlorophyll loss in both wild-type and

mutant leaves. Although *arf2-8* leaves senesced more slowly than Columbia leaves, the relative delay in chlorophyll loss caused by exogenous cytokinin appeared similar for both genotypes. It therefore seems unlikely that delayed senescence of *arf2-8* leaves was due to alterations in cytokinin sensitivity.

nph4 and *arf19* mutations enhance *arf2* phenotypes

NPH4/ARF7 and *ARF19* are closely related and function redundantly in controlling tropisms, lateral root initiation and leaf expansion (Okushima et al., 2005a; Wilmoth et al., 2005). Expression profiling indicates that like *ARF2*, both these genes are induced in senescing leaves (Lin and Wu, 2004) and this was confirmed by RNA gel blot analysis (Fig. 8C). *NPH4/ARF7* mRNA levels in *arf2-8* leaves were approximately twofold higher than in Columbia leaves at the start of the experiment. Although mutations in *NPH4/ARF7* and *ARF19* did not affect senescence of rosette leaves or floral organs on their own, they enhanced the effects of the *arf2-8* mutation. Eight days after detachment, *arf2-8* leaves retained 49% of their chlorophyll, whereas *arf2-8 nph4-1 arf19-4* leaves retained 65% (Fig. 8A). Furthermore, whereas *arf2-8* flowers shed their organs at position 9, *arf2-8 nph4-1 arf19-4* flowers shed at position 13 (Fig. 8B). These experiments do not indicate whether *NPH4/ARF7* and *ARF19* are both required for the delay in senescence. However, both genes were induced by senescence and they act redundantly in other aspects (Okushima et al., 2005a; Wilmoth et al., 2005), so it seems likely that they both function in this respect.

In addition, the anthers of *arf2-8 nph4-1 arf19-4* flowers did

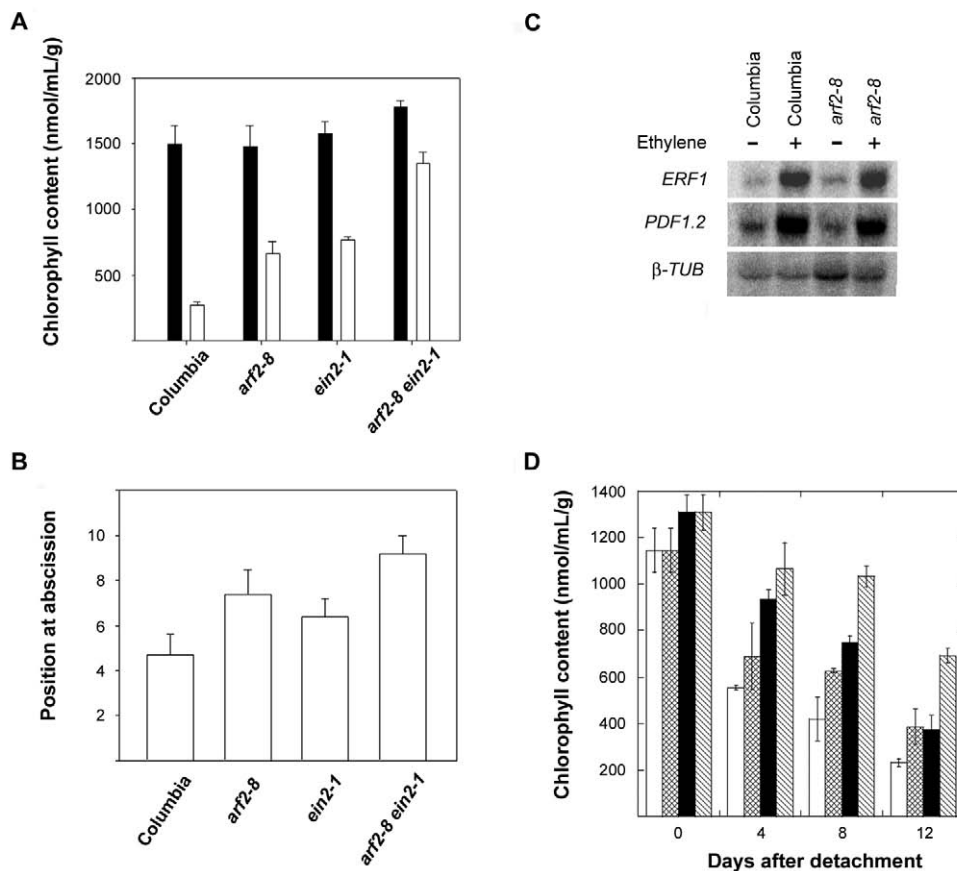


Fig. 7. Effects of ethylene and cytokinin signaling on *arf2-8* plants. (A) Chlorophyll levels in detached leaves incubated in the dark for 8 days. Black bars represent chlorophyll levels just after detachment and white bars represent chlorophyll levels of leaves incubated in the dark for 8 days. Error bars indicate s.d. ($n=6$). (B) Average position at abscission where position 1 equals the first opened (stage 13) flower and flowers are numbered basipetally. Error bars indicate s.d. ($n>20$). (C) Northern blot analysis of ethylene-induced gene expression of *arf2-8* plants. Six-week-old plants were incubated in airtight containers with either 10 ppm ethylene or air. RNA was extracted from whole rosettes. (D) Chlorophyll levels in cytokinin-treated detached leaves. Detached leaves were floated on water supplemented with 100 nM 6-benzyladenine or on a DMSO mock control. White, Columbia; cross hatched, Columbia+BA; black, *arf2-8*; hatched, *arf2-8*+BA. Error bars indicate s.d. ($n=6$).

not dehisce and the plants were therefore male sterile. Unlike *arf6 arf8* flowers (Nagpal et al., 2005), jasmonic acid did not rescue the anther dehiscence phenotype. Whereas *nph4* and *arf19* enhanced the delayed senescence of *arf2*, the *arf2* mutation did not affect the leaf expansion or gravitropism phenotypes of *nph4 arf19* plants.

Discussion

ARF2 has pleiotropic effects on plant development

Our data show that *arf2* plants have delays in rosette leaf senescence, induction of flowering, stamen elongation, floral organ abscission and silique ripening. Additionally, other workers have found that *arf2* mutant plants have delayed apical hook opening, enlarged seeds, stems and cotyledons, and elongated hypocotyls under red light (Li et al., 2004, Okushima et al., 2005b; Schruff et al., 2005). These diverse phenotypes indicate that ARF2 functions at multiple stages in the life cycle of *Arabidopsis*.

Does ARF2 promote these phenotypes by a common mechanism? The abscission of floral organs and silique dehiscence phenotypes involve delays in senescence and may therefore be regulated by similar mechanisms as rosette leaf senescence. More generally, ARF2 may control production of some metabolic signal which in turn regulates these transitions as well as flowering and stamen development. Leaf and lateral root initiation and the timing of abaxial trichome production proceeded normally in *arf2* plants (data not shown), so the developmental delays seen are not likely due to slower overall growth or to slower phase change. Thus, if ARF2 controls these transitions via a common signal, it is unlikely to be a growth-limiting substance.

An alternative model is that ARF2 acts through distinct mechanisms in different tissues and/or at different developmental stages. Consistent with this idea, ARF2 is a target of ethylene signaling in etiolated seedlings (Li et al., 2004), but appears to act independently of ethylene in senescing leaves and flowers. In addition, *arf2* mutants have abnormally large inflorescence stems and ovule integuments caused by increased cell numbers, suggesting that ARF2 may normally inhibit growth and cell division in ovules and stems (Schruff et al., 2005; Okushima et al., 2005b). However, these phenotypes might have arisen if *arf2* mutations prolong the period of stem and ovule growth, thereby allowing time for extra cell divisions to occur. In other tissues, increasing the duration of a 'growth' period might in effect delay the transition to a subsequent stage, as we suggest occurs in leaves and flowers of *arf2* mutants.

ARF1 and ARF2 have both distinct and overlapping functions

Of the 22 ARF genes in *Arabidopsis thaliana*, ARF2 is most similar to ARF1 (Remington et al., 2004). However, *arf1* plants had no defects in senescence, flowering time, abscission of floral organs, fertility, auxin-mediated lateral root initiation, auxin-inhibited root elongation, hypocotyl elongation in response to different light regimes, gravitropism, phototropism or shoot branching (data not shown). Although *arf1* mutations did not confer phenotypes on their own, they did enhance late flowering, floral organ abscission and stamen elongation phenotypes of *arf2-8* and

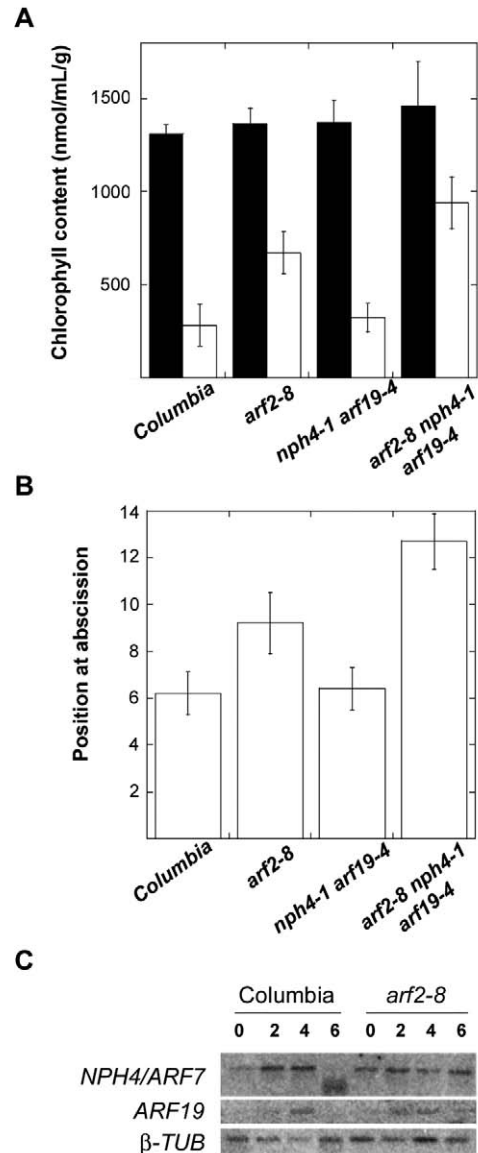


Fig. 8. Phenotypes of *arf2-8 nph4-1 arf19-4* plants. (A) Chlorophyll levels in detached leaves incubated in the dark for 8 days. Black bars represent chlorophyll levels just after detachment and white bars represent chlorophyll levels of leaves incubated in the dark for 8 days. Error bars indicate s.d. ($n=6$). (B) Average position at abscission where position 1 equals the first opened (stage 13) flower and flowers are numbered basipetally. Error bars indicate s.d. ($n=25$). (C) Northern blot analysis of *NPH4/ARF7* and *ARF19* expression in senescing leaves. Leaves from Columbia and *arf2-8* plants were detached, placed in the dark for various time periods and used for mRNA extraction.

Ws-0 dsARF2 plants, and the delayed leaf senescence of *Ws-0 dsARF2* plants. Similarly, *arf1* mutations enhanced the effects of *arf2* mutations on apical hook formation (Li et al., 2004). These results indicate that ARF1 and ARF2 have some functions in common.

Despite these similarities, results from gene expression analyses suggest that ARF1 and ARF2 also have distinct activities. In particular, *arf1-4* mutant plants had increased

expression of auxin responsive genes in seedlings, developing stamens and in the abscission zone at the base of floral organs, whereas *arf2* and *dsARF2* plants had normal expression of these genes. ARF1 can repress auxin-induced gene expression in transient assays (Ulmasov et al., 1999a), and the increased expression of *IAA3* and *IAA7* in *arf1-4* flowers suggests that ARF1 also represses gene expression in planta.

The ethylene precursor ACC increased expression of the auxin-responsive reporter gene *DR5::GUS* (Ulmasov et al., 1997b) in etiolated *arf2* seedlings more than in wild-type plants (Li et al., 2004), showing that increased expression of auxin-regulated genes in *arf2* plants may occur in response to certain stimuli. However, we and others have failed to detect consistent alterations in auxin-induced gene expression in *arf2* mutants (Fig. 6S) (Okushima et al., 2005b) (M. C. Schruff, M. Spielman and R. J. Scott, personal communication), suggesting that ARF2 does not suppress auxin-induced gene expression in a general manner. It therefore seems likely that ARF2 function involves additional mechanisms, and ARF2 may not conform to the canonical auxin response model. Phylogenetic studies indicate that *ARF1* and *ARF2* diverged prior to the monocot-dicot split (Remington et al., 2004; Sato et al., 2001) and thus may have had ample time to evolve distinct biochemical activities.

Activating ARFs also affect senescence and abscission

Mutations in *NPH4/ARF7* and *ARF19* did not affect senescence on their own, but enhanced *arf2-8* phenotypes as did mutations in *ARF1*. Unlike ARF1, which represses gene expression, *NPH4/ARF7* and *ARF19* activate gene expression in protoplast assays and in vivo (Okushima et al., 2005a; Wilmoth et al., 2005). Expression of *ARF2*, *NPH4/ARF7* and *ARF19* increased in response to senescence, and promoter::GUS fusions to all three (as well as the *P_{ARF2}::GUS:ARF2* protein fusion) were expressed in leaf vasculature (Okushima et al., 2005a; Wilmoth et al., 2005). In addition, *ARF1*, *ARF2*, *NPH4/ARF7* and *ARF19* are all expressed at the base of the flower, including the abscission zone (this study) (Wilmoth et al., 2005). These results suggest that ARF1, ARF2, *NPH4/ARF7* and *ARF19* are all present in the same tissues and might interact together in the same cells. MADS-box proteins may regulate floral organ identity in higher-order complexes (Honma and Goto, 2001), and ARFs may interact analogously. For example, ARF2 might recruit activating ARFs to promoters that they would otherwise recognize poorly. In protoplast assays, the ability of ARFs to regulate promoter activity depended on the arrangement of AuxREs, suggesting that ARFs may indeed have different specificities for different promoters (Tiwari et al., 2003). Alternatively, different ARFs may target different promoters and thus affect different aspects of senescence. Aux/IAA proteins can inhibit transcriptional activation by activating ARFs (Tiwari et al., 2003; Tiwari et al., 2004), potentially adding further regulatory inputs.

Auxin and senescence

Classical studies have correlated auxin levels with senescence and abscission (reviewed in Addicott, 1982; Nooden and Leopold, 1988; Sexton and Roberts, 1982). In bean leaves, a gradient of auxin levels was detected between the leaf blade

and the stalk. Auxin levels declined with leaf age and senescence occurred when auxin levels between the leaf and stalk were approximately equal (Shoji et al., 1951). Application of IAA to the distal end of abscission zone explants delayed abscission, while addition to the proximal end promoted abscission (Addicott and Lynch, 1951). This suggests that changes in auxin gradients may signal the onset of or enhance senescence and may explain the observation that treating whole plants with auxin had little effect on abscission (Addicott et al., 1955). These conclusions also raise the possibility that ARF1, ARF2, *NPH4/ARF7* and *ARF19* may interact in some way to read auxin gradients.

Comparisons to other delayed senescence and abscission mutants of *Arabidopsis*

Transgenic plants that overexpress the MADS transcription factor gene *AGL15* also had delayed flowering, floral organ abscission and fruit ripening (Fernandez et al., 2000; Fang and Fernandez, 2002). Those authors speculated that *AGL15* may be involved in maintaining plants in a juvenile state. As ARF2 accelerates the same transitions, ARF2 may therefore antagonize *AGL15* to control the developmental progression of plant aging. However, *arf2* plants show some abnormalities that *35S::AGL15* plants do not, such as delayed leaf senescence and reduced fertility. *arf2* mutants had normal *AGL15* expression levels, and the *P_{ARF2}::GUS* fusion was expressed normally in *35S::AGL15* plants (data not shown), suggesting that *ARF2* and *AGL15* do not regulate the expression of one another.

A number of other *Arabidopsis* mutant plants also have delays in floral organ abscission (Jinn et al., 2000; Patterson and Bleecker, 2004; Butenko et al., 2003; Fernandez et al., 2000), but differ from *arf2* plants in significant respects. Whereas abscission is delayed by three or four flowers in *arf2* plants, the *inflorescence deficient in abscission (ida)* mutant has a complete loss of floral organ shedding (Butenko et al., 2003). The *ida* gene is predicted to encode a 77 amino acid peptide that may act as a ligand for a protein such as HAESA, a receptor-like protein kinase whose antisense suppression resulted in a delay of floral organ abscission (Jinn et al., 2000). In a screen for delayed floral organ abscission (*dab*) mutants, three loci were identified (Patterson and Bleecker, 2004). Sepals of *dab2* and *dab3* plants turned yellow prior to detachment, but *dab1* sepals, like *arf2* sepals, remained green and turgid. However, all other aspects of plant growth appeared normal in these plants, whereas *arf2* plants had multiple defects throughout plant development.

Arabidopsis mutants displaying delays in leaf senescence (for a review, see Lim et al., 2003) include plants with mutations in hormone signaling pathways such as ethylene (Grbic and Bleecker, 1995) and cytokinin (To et al., 2004). *arf2-8* plants were as sensitive to exogenous cytokinin as wild-type plants, implying that ARF2 affects senescence independently of cytokinin. The ethylene response mutant *ein2* has a phenotype similar to that of *arf2* in both leaf senescence and delayed floral organ abscission. Although ethylene and *ARF2* each promoted senescence, they have opposing effects on apical hook formation, and in etiolated seedlings ethylene promotes the degradation of ARF2 protein (Li et al., 2004). However, in mature plants, *ARF2* appears to function independently of the ethylene signaling pathway as *arf2* and

ein2 mutations had additive effects on leaf senescence and floral organ abscission. Ethylene levels increase in senescing leaves, yet *ARF2* transcript and GUS::*ARF2* fusion protein increased in detached leaves undergoing dark-induced senescence. It is therefore unlikely that ethylene causes the depletion of *ARF2* in mature leaves. Taken together, these results suggest that, unlike during apical hook formation, *ARF2* does not act downstream of ethylene in the control of leaf senescence or floral organ abscission.

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