**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 expressions 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 (Ulmason 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 (Wilmuth 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 and ARF19, 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; Wilmuth 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 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.

**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
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 (hs1) 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 hs1 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 stems, siliques and cotyledons, and elongated hypocotyls under red light, but did not find significant effects on auxin-responsive gene expression (Schurr et al., 2005; Okushima et al., 2005a).

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 mutants 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 line was characterized in greater detail. In addition, a dsRNAi line was derived from pools of T-DNA insertion lines, using ARFdIV-1 and ARF1 promoter-GUS-nos fragment was excised with EcoRI and cloned into pZIP211. PARF2::GUS lines were created by amplifying a 2.5 kb fragment from BAC AB016880 DNA using the primers: 5'-CACACAAATGTCGAGATGACTTGGTGTCGTA-3' and 5'-CGCAGATTTCAAGCTGACAT-3'. Seven plant lines per construct were analyzed for GUS activity and representative lines were chosen for further analysis. PARF2::GUS and PIAA7::GUS lines were described previously (Tian et al., 2002). Plant tissue was stained for GUS activity as described previously (Wilmot 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 oligot(dT)15 Dynabeads according to manufacturers' instructions (Dynal, 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'-CCAAATGTCGAGATGACTTGGTGTCGTA-3' and 5'-GCAAATGACGCCTTGGTTGGC-3'; ARF2, 5'-GAAAGGTATGCGAGATTCCGAGGGGAGATGAGTTCGTTGGC-3' and 5'-GAGATTTCAAGCTGACAT-3'; AI3, 5'-CTTCATACATCACGAGCCTTCTTCA-3' and 5'-GGGGATGTTCAGTTTTCTCTTCA-3'; IA7, 5'-CAAGAATACATGCAAGCACAAGG-3' and 5'-GCTTTAGAGAAATTTAAGAGGTG-3'; SAG12, 5'-GATTGTTTGGGGCTGTCGTA-3' and 5'-CTTTCATGCGCAGACACATGTC-3'; IA2, 5'-CTTATGATCAGAGAAAGCTGAGAATTG-3' and 5'-ATCTCATGTATCTTACATCAAACCTTC-3'; and 5'-GAAGGTATGCGAGATTCCGAGGGGAGATGAGTTCGTTGGC-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 μm) 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.
and is therefore an excellent marker gene for development-mediated senescence. Leaves from Columbia plants that had been placed in the dark accumulated \textit{SAG12} mRNA starting after 2 days, and \textit{SAG12} levels peaked after 4 days (Fig. 3C). \textit{arf2-8} leaves accumulated very little \textit{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, \textit{SAG12} expression levels and chlorophyll loss were similar in \textit{Ws-0} and \textit{arf1-4} plants, suggesting that \textit{ARF1} on its own has little effect on senescence.

In wild-type plants, levels of \textit{ARF2} mRNA also increased in detached leaves following dark treatment, with a time course similar to that of \textit{SAG12} mRNA (Fig. 3C). Microarray studies also indicated that \textit{ARF2} mRNA increased during senescence (Lin and Wu, 2004). We examined \textit{PARF2::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 \textit{PARF2::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 \textit{ARF2}, \textit{ARF1} mRNA decreased as senescence progressed in both Columbia and \textit{arf2-8} plants (Fig. 3C). \textit{ARF1} mRNA levels were quantified in three independent blots and were found to be slightly higher (2.7±0.9-fold) in freshly detached \textit{arf2} leaves when compared with Columbia leaves. These patterns suggest that \textit{ARF1} has a different function than does \textit{ARF2} during wild-type leaf senescence.

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

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

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arl1-5 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 sepal, 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 Bleecker, 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 siliques at abscission. In Ws-0 and Columbia, flowers shed their perianth organs when the siliques 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 siliques was 10.6 and 10.2 mm, respectively (Fig. 5F,G). In addition to being shed later than wild-type sepal, sepal of arf2 and arf1 arf2 plants remained green and turgid until detachment, whereas wild-type sepal 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 siliques 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.
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<sub>ARF1</sub>:GUS and P<sub>ARF2</sub>: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<sub>ARF1</sub>:GUS and P<sub>ARF2</sub>: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<sub>ARF2</sub>:GUS::ARF2 plants (Li et al., 2004) showed similar staining patterns as P<sub>ARF2</sub>:GUS plants (data not shown), suggesting that the P<sub>ARF2</sub>: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).

Table 1. Measurements of wild-type and mutant plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of rosette leaves before bolting</th>
<th>Time to bolting (days)</th>
<th>Stamen length of 2 mm bud (mm)</th>
<th>Stamen length at anthesis</th>
<th>Carpel length at anthesis</th>
<th>Average position at abscission</th>
<th>Siliq length at floral organ abscission (mm)</th>
<th>Time to silique dehiscence (DAP)</th>
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<tr>
<td></td>
<td>12.2±1.8 (12)</td>
<td>26.2±0.9 (12)</td>
<td>1.3±0.1 (20)</td>
<td>2.3±0.2 (25)</td>
<td>6.5±1.1 (32)</td>
<td>4.5±0.7 (136)</td>
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<td></td>
<td>12.5±1.2 (11)</td>
<td>26.7±1.8 (11)</td>
<td>1.3±0.1 (20)</td>
<td>2.3±0.2 (25)</td>
<td>5.9±0.8 (30)</td>
<td>4.5±0.8 (118)</td>
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<td>15.0±1.4 (9)</td>
<td>31.1±0.9 (9)†</td>
<td>1.4±0.1 (20)</td>
<td>2.5±0.2 (25)</td>
<td>8.0±0.7 (29)†</td>
<td>10.0±1.8 (55)†</td>
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<td>23.4±2.2 (31)</td>
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<td>16.2±1.0 (7)</td>
<td>31.3±1.3 (7)</td>
<td>1.3±0.1 (20)</td>
<td>2.4±0.2 (25)</td>
<td>9.0±0.8 (25)†</td>
<td>10.6±1.9 (65)</td>
<td>18.9±1.3 (28)</td>
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<td>2.4±0.2 (25)</td>
<td>6.1±0.9 (25)†</td>
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<td>23.7±0.6 (10)</td>
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<td>10.2±2.1 (94)</td>
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Values given are mean±s.d. (n)

* 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.

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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. 6J,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. 6L), and P_{IAA7}::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...
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.

PSAG12::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...
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.](image)

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 \textit{arf}2 and \textit{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 \textit{IAA3} and \textit{IAA7} in \textit{arf1-4} flowers suggests that ARF1 also represses gene expression in planta.

The ethylene precursor ACC increased expression of the auxin-responsive reporter gene \textit{DR5}::\textit{GUS} (Ulmasov et al., 1997b) in etiolated \textit{arf2} seedlings more than in wild-type plants (Li et al., 2004), showing that increased expression of auxin-regulated genes in \textit{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 \textit{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 \textit{ARF1} and \textit{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.

\textbf{Activating ARFs also affect senescence and abscission}

Mutations in \textit{NPH4/ARF7} and \textit{ARF19} did not affect senescence on their own, but enhanced \textit{arf2}-8 phenotypes as did mutations in \textit{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 \textit{ARF2}, \textit{NPH4/ARF7} and \textit{ARF19} increased in response to senescence, and promoter::\textit{GUS} fusions to all three (as well as the \textit{P}\textit{ARF2}::\textit{GUS}:\textit{ARF2} protein fusion) were expressed in leaf vasculature (Okushima et al., 2005a; Wilmoth et al., 2005). In addition, \textit{ARF1}, \textit{ARF2}, \textit{NPH4/ARF7} and \textit{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.

\textbf{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.

\textbf{Comparisons to other delayed senescence and abscission mutants of Arabidopsis}

Transgenic plants that overexpress the MADS transcription factor gene \textit{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, \textit{arf2} plants show some abnormalities that \textit{35S::AGL15} plants do not, such as delayed leaf senescence and reduced fertility. \textit{arf2} mutants had normal AGL15 expression levels, and the \textit{P}\textit{ARF2}::\textit{GUS} fusion was expressed normally in \textit{35S::AGL15} plants (data not shown), suggesting that \textit{ARF2} and \textit{AGL15} do not regulate the expression of one another.

A number of other \textit{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 \textit{arf2} plants in significant respects. Whereas abscission is delayed by three or four flowers in \textit{arf2} plants, the \textit{inflorescence deficient in abscission (ida)} mutant has a complete loss of floral organ shedding (Butenko et al., 2003). The \textit{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 (\textit{dab}) mutants, three loci were identified (Patterson and Bleecker, 2004). Sepals of \textit{dab2} and \textit{dab3} plants turned yellow prior to detachment, but \textit{dabl} sepal, like \textit{arf2} sepal, remained green and turgid. However, all other aspects of plant growth appeared normal in these plants, whereas \textit{arf2} plants had multiple defects throughout plant development.

\textit{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). \textit{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 \textit{ein2} has a phenotype similar to that of \textit{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 \textit{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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