Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function

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Summary

The phytohormone gibberellin (GA) regulates the development and fertility of *Arabidopsis* flowers. The mature flowers of GA-deficient mutant plants typically exhibit reduced elongation growth of petals and stamens. In addition, GA-deficiency blocks anther development, resulting in male sterility. Previous analyses have shown that GA promotes the elongation of plant organs by opposing the function of the DELLA proteins, a family of nuclear growth repressors. However, it was not clear that the DELLA proteins are involved in the GA-regulation of stamen and anther development. We show that GA regulates cell elongation rather than cell division during *Arabidopsis* stamen filament elongation. In addition, GA regulates the cellular developmental pathway of anthers leading from microspore to mature pollen grain. Genetic analysis shows that the *Arabidopsis* DELLA proteins RGA and RGL2 jointly repress petal, stamen and anther development in GA-deficient plants, and that this function is enhanced by RGL1 activity. GA thus promotes *Arabidopsis* petal, stamen and anther development by opposing the function of the DELLA proteins RGA, RGL1 and RGL2.

Key words: Gibberellin, DELLA proteins, Stamen development, Floral development

Introduction

Gibberellin (GA) is an endogenous regulator of plant growth (Richards et al., 2001). Severely GA-deficient *Arabidopsis* mutants, such as *ga1-3*, exhibit retarded vegetative growth of shoots (Koornneef and van der Veen, 1980; Peng and Harberd, 1997; King et al., 2001) and roots (Fu and Harberd, 2003). In addition, the development of floral organs, especially petals and stamens, is impaired in GA-deficient mutants, while retarded anther development results in male-sterility owing to a lack of mature pollen (Wilson et al., 1992; Goto and Pharis, 1999). All of the GA-deficient mutant floral phenotypes of *ga1-3* can be restored to normal by application of exogenous GA (Koornneef and van der Veen, 1980), demonstrating that GA regulates floral development (Pharis and King, 1985). Anther development is GA dependent in a range of plant species additional to *Arabidopsis* (Nester and Zeevaart, 1988; Jacobsen and Olszewski, 1991), suggesting that GA is a general regulator of floral development.

The histology of anther development (microsporogenesis) is well documented (Goldberg et al., 1993; McCormick, 1993; Sanders et al., 1999). In addition, the study of mutants exhibiting stage-specific defects in microsporogenesis and pollen release has further advanced our understanding of the process (Preuss et al., 1993; Sanders et al., 1999; Park and Twell, 2001; Azumi et al., 2002; Steiner-Lange et al., 2003; Unte et al., 2003). Although little is known about how GA controls stamen filament elongation, anther development or microsporogenesis, there have been previous suggestions that GA-signalling components may modulate these processes. For example, overexpression of SPY (an *Arabidopsis* GA-signalling component) (Jacobsen et al., 1996) inhibits post-meiotic anther development in petunia (Izhaki et al., 2002). Furthermore, transgenic expression of wild-type or mutant forms of GAI (another *Arabidopsis* GA-signalling component, see below) can retard stamen elongation and induce male-sterility in tobacco and *Arabidopsis* (Huang et al., 2003; Hynes et al., 2003). By contrast, infertility caused by impaired floral development is also a characteristic of mutants lacking the rice or barley DELLA proteins SLR1 or SLN1 (Ikeda et al., 2001; Chandler et al., 2002). Despite these various reports, the mechanism via which GA regulates petal, stamen and anther development remained unclear.

Recent advances have enabled the identification of a family of proteins homologous to *Arabidopsis* GAI and RGA (Peng et al., 1997; Silverstone et al., 1998) that are crucial for the regulation of plant stem elongation growth in response to GA (Peng et al., 1999; Ikeda et al., 2001; Boss and Thomas, 2002; Chandler et al., 2002). These proteins belong to the DELLA family (Fleck et al., 2002), a subfamily of the GRAS family of putative transcriptional regulators (Pysh et al., 1999; Richards

et al., 2000). The *Arabidopsis* genome encodes five distinct DELLA proteins (Lee et al., 2002). Genetic suppression studies have shown that GAI and RGA functions overlap in the repression of plant stem growth (Dill and Sun, 2001; King et al., 2001). Further studies showed that while RGL2 controls seed germination (Lee et al., 2002), RGL1 may control stem elongation as well as seed germination (Wen and Chang, 2002). Although *GAI*, *RGA*, *RGL1* and *RGL2* are all expressed in developing inflorescences (Lee et al., 2002), no obvious suppression of *ga1-3* floral phenotype was observed in *ga1-3* mutants lacking GAI, RGA, GAI and RGA, or RGL2 (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002). However, a transgenic *RGL1* loss-of-function line was resistant to the arrest of floral organ development induced by paclobutrazol (PAC, an inhibitor of GA biosynthesis) (Wen and Chang, 2002), suggesting that RGL1 might play a role in regulating floral development. These observations underscore the importance of determining systematically the respective roles of the various DELLA proteins in GA-mediated regulation of *Arabidopsis* petal and stamen development.

In this report, we describe experiments addressing two questions. First, we characterized *ga1-3* floral development to determine at which stage of stamen/anther development GA-deficiency causes developmental arrest. Second, we used novel combinations of loss-of-function mutations to determine if DELLA proteins are repressors of stamen filament elongation and microsporogenesis. Our results show that GA is crucial both for cell elongation during stamen elongation and for the developmental progression from microspore to mature pollen grain during pollen development. We also show that the DELLA proteins RGA, RGL1 and RGL2 work together to repress stamen and anther development in GA-deficient plants.

Materials and methods

Plant growth conditions, genetic nomenclature, plant materials

Plants were grown as described previously (Lee at al., 2002), or in controlled long (LD)- and short (SD)-day growth conditions (Peng and Harberd, 1997). Genotypes are written in capital italics (e.g *GAI*), mutant genotypes in lowercase (e.g. *gai-t6*). Polypeptide gene products are written in nonitalic capitals (e.g. GAI). Mutant lines (Landsberg *erecta* background) *rgl1-1*, *rgl2-1*, *gai-t6*, *rga-t2* and *ga1-3* were as described (Peng et al., 2002; Lee et al., 2002). The *ga1-3* lines lacking various combinations of RGL1, RGL2, GAI or RGA were as described previously (King et al., 2001; Lee et al., 2002), or made via cross-pollination. Primer pairs used for genotype verification were as described previously (Lee et al., 2002). For anatomical analysis of *ga1-3*, seeds were chilled for 7 days, following which the seed coat was manually removed and the seed sown on soil.

Histology and in situ hybridization

For scanning electron microscopy, individual flower buds from fresh wild-type or mutant inflorescences were dissected; outer organs were removed using stainless steel needles. Buds were attached to a mounting plate, plunged into liquid nitrogen, quickly transferred to a specimen chamber and scanned at 10 KV (JSM-5310LV, JEOL, Japan). Pollen grains were mounted on scanning electron microscopy stubs and coated with gold using previously described techniques (Bozzola and Russell, 1999). For anther sectioning, fresh inflorescences were fixed in formalin-acetic acid-alcohol (FAA) fixative buffer at 4°C overnight followed by dehydration steps and subsequent embedding in Jung Historesin (Leica). Sections (2.5 μm) were made using a Leica RM 2055 microtome and stained with 0.25% Toluidine Blue O (Sigma). DAPI staining of pollen grain nuclei was performed as described (Chen and McCormick, 1996) and pollen numbers were counted under a microscope (Leica DM RXA2) with 40× or 20× objectives. Color photos were taken using a Spot Insight QE digital camera (Diagnostic Instruments). The *ATA7* and *SDS* antisense and sense probes were synthesized from the pMC1577 and pMC2317 plasmids, respectively (Zhao et al., 2002) and in situ hybridisation was performed as described previously (Luo et al., 1996). Callose staining and chromosome spread analysis of meiotic stages were as described (Regan and Moffatt, 1990; Ross et al., 1996).

Results

GA regulates epidermal cell elongation during stamen elongation

To determine the developmental stage at which *ga1-3* flower buds become arrested, the relative growth of stamens versus gynoecium in inflorescence flower buds [starting with the outmost (oldest) and ending with the innermost dissectable bud] was compared in *ga1-3* and wild type via scanning electron microscopy (SEM). All floral organs were properly initiated in *ga1-3*, and appeared to develop normally, though only to around floral stage 10 (Fig. 1A) [floral stages as defined elsewhere (Smyth et al., 1990; Bowman, 1994)]. Subsequent petal and stamen development beyond stage 10 was arrested in *ga1-3* (compare wild type at floral stage 13 with *ga1-3*; Fig. 1A). The ultimate stamens and pistils of *ga1-3* flower buds were much shorter than the mature stamens and pistils of the wild-type control (Fig. 1B). In addition, *ga1-3* had a stronger effect on stamen filament length than on pistil length: the arrest of stamen development resulted in significantly shorter stamens versus pistils in *ga1-3* (Fig. 1B). SEM of stamen filament epidermal cells indicated that the arrest of stamen filament growth in *ga1-3* was due to reduced cell length (Fig. 1C), rather than to a reduction in cell number (Fig. 1D).

ga1-3 plants fail to produce tricellular pollen grains

In *Arabidopsis*, the anther consists of four lobes, each of identical cell-type architecture, each derived from archesporial cells. Microsporogenesis initiates within the reproductive locule of each lobe. The sporogenous cells divide to generate microspore mother cells (MMC). Subsequently, the MMC undergo meiosis to generate tetrads of haploid microspores (MSp). The MSp are released from the tetrads and undergo two rounds of cell division to form mature tricellular pollen grains (Sanders et al., 1999).

The surface structure of the mature pollen grains released by wild-type plants was compared with that of immature pollen grains manually dissected from *ga1-3* anther locules. All wild-type pollen grains were oval shaped with long indented lines. Very few oval shaped pollen grains were observed in *ga1-3*. Instead, in most cases, the immature pollen grains from *ga1-3* plants were spherical in shape (Fig. 2A). Wild-type and *ga1-3* anthers were dissected and stained with DAPI. As expected, the mature pollen grains from wild-type plants were tricellular, and contained three nuclei (Fig. 2B). However, in most cases (see Discussion), fewer than 10% of the developing grains examined in *ga1-3* pollen sacs were found to be bicellular/tricellular (Fig. 2B,C). In fact, about 48% of *ga1-3* pollen grains contained only a single nucleus

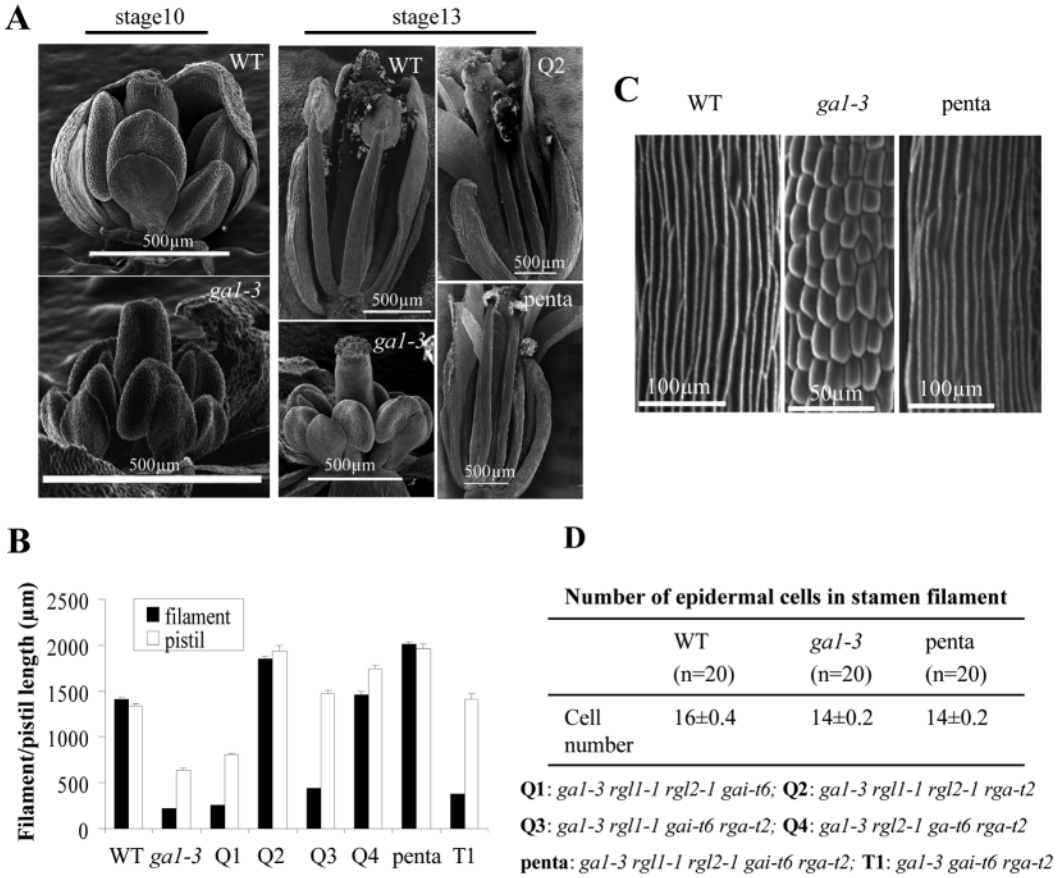


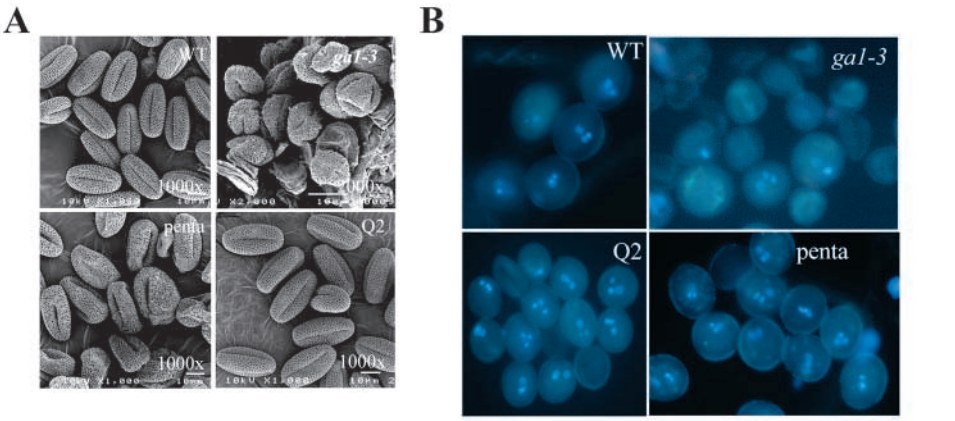
Fig. 1. GA regulates stamen filament length via control of cell elongation. (A) SEMs of wild-type flowers at floral stages 10 and 13 are shown. Petals and stamens from the most advanced flower (middle bottom) in *ga1-3* resembled the wild-type flower at stage 10 (top left). Stamen filament elongation is dramatically increased in *ga1-3* plants lacking RGL1, RGL2 and RGA (Q2) and in *ga1-3* plants lacking RGL1, RGL2, GAI and RGA (penta) compared with the wild type. Both Q2 and penta lines produced visible pollen grains. (B) Comparison of stamen and pistil lengths among different genotypes. Filament and pistil lengths were measured from SEM pictures (n=20). (C) SEM of stamen filament epidermal cells. *ga1-3* stamen filaments have relatively short epidermal cells compared with those of wild type; cell length was restored in stamen filaments of *ga1-3* plants lacking RGL1, RGL2, GAI and RGA (penta). Stamen filament segments shown were all from the middle part of the filament. (D) Average number of epidermal cells per stamen filament in wild type, *ga1-3* and *ga1-3* lacking RGL1, RGL2, GAI and RGA (penta).

and 46% had no nucleus. Clearly, *ga1-3* fails to produce mature pollen, and this probably results from an arrest or impairment in pollen development prior to or during pollen mitosis in *ga1-3* (Fig. 2C).

Microsporogenesis is arrested before pollen mitosis in ga1-3

As in other plant species, microsporogenesis in *Arabidopsis* is a highly programmed process, and can be divided into 14 stages at the cellular level (Sanders et al., 1999). In wild type, floral stage 10 corresponds to stage 7-8 of microsporogenesis (pre-mitosis) (Bowman, 1994). As shown above, petal and stamen growth arrests at around floral stage 10 in *ga1-3*, suggesting that microsporogenesis may arrest prior to pollen mitosis in this mutant. To define the specific stage of microsporogenesis arrested in the *ga1-3* mutant, we compared transverse sections of anthers from *ga1-3* and wild type. No obvious differences were observed between *ga1-3* and wild type up to microsporogenesis stage 7, when tetrads are formed (Fig. 3). However, after stage 7, it appeared that the microspores in *ga1-3* were not properly released and that the *ga1-3* pollen sacs failed to expand. In addition, the *ga1-3* tapetum remained at the vacuole stage and then subsequently degenerated together with the microspores and pollen sacs (Fig. 3). As a result, the later stages of microsporogenesis (stages 9-12) could not be convincingly distinguished in *ga1-3*, suggesting that microsporogenesis is arrested at stages 7-8 (prior to pollen mitosis), thus preventing the formation of mature pollen.

To confirm that microsporogenesis was arrested prior to pollen mitosis in *ga1-3*, we used the gene expression markers *SDS* and *ATA7*. *SDS* is a marker gene that is specifically expressed in microsporocytes (Azumi et al., 2002). In situ hybridization analysis revealed near-identical expression patterns for *SDS* in the anthers of *ga1-3* mutants and wild type, suggesting that the early stages of microsporogenesis are not affected in *ga1-3* (Fig. 4A). In fact, chromosome spread experiments confirmed that meiosis in *ga1-3* was successfully accomplished, resulting in the formation of tetrads (Fig. 4B). However, Aniline Blue staining showed that, although the callose wall was formed successfully, the tetrads in *ga1-3* anthers were not properly separated from one another, as is seen in wild type (Fig. 4C). *ATA7* is an early tapetum-specific molecular marker (Rubinelli, 1998). As expected, *ATA7* was highly expressed in wild type between stages 7 and 9 (Fig. 4A). The *ATA7* signal then gradually disappeared during the later stages of microsporogenesis, although pollen mitosis was occurring (data not shown). *ATA7* was also strongly expressed in *ga1-3* anthers (Fig. 4A), implying that tapetum initiation is not detectably affected by GA deficiency. However, in most cases, the *ga1*-3 pollen sacs marked by *ATA7* were irregular in shape and contained microspores that had become deformed and severely degenerate at a developmental stage prior to the disappearance of the *ATA7* signal (Fig. 4A).



C

DAPI staining to examine tricellular pollens

Genotype	2-3 nuclei (%)	1 nuclei (%)	No nuclei (%)	Pollen numbers examined	Number of flowers
WT	97.5	0.7	1.8	2159	6
ga1-3	6.5	48	45.5	4408	27
Q1	28.3	7.6	64.1	2768	20
Q2	78.9	0.3	20.8	2635	12
Q3	33.9	14.5	51.6	4825	40
Q4	58	5.2	36.8	2906	24
penta	88	0.1	11.9	1216	8

Q1: *ga1-3* lacking RGL1, RGL2 and GAI; Q2: *ga1-3* lacking RGL1, RGL2 and RGA; Q3: *ga1-3* lacking RGL1, GAI and RGA; Q4: *ga1-3* lacking RGL2, GAI and RGA; penta: *ga1-3* lacking RGL1, RGL2, GAI and RGA

Fig. 2. *ga1-3* plants fail to produce tricellular pollen grains. (A) Pollen grains from various genotypes examined by SEM. Pollen grains from *ga1-3* plants lacking RGL1, RGL2 and RGA (Q2), or RGL2 and RGA (not shown) were almost identical to wild-type pollen grains, being oval shaped with long indented lines on the surface. In *ga1-3* plants, the cells contained in a locule appeared to remain as microspores, being round and, in some cases, with short indented lines on the wall surface. Pollen grains from *ga1-3* plants lacking RGL1, RGL2, GAI and RGA (penta) were similar to wild-type pollen grains but were slightly more wrinkled in appearance. (B) DAPI staining showed that pollen grains from *ga1-3* plants lacking RGL1, RGL2 and RGA (Q2), or RGL1, RGL2, GAI and RGA (penta) are tricellular (as in the wild-type control). By contrast, the majority of the cells in the anther locule of *ga1-3* had either no detectable nucleus or only a single condensed nucleus whereas only ~6% had two or more than two nuclei. (C) Frequencies of tricellular pollen grains in anther locules of various genotypes as revealed by DAPI staining as shown in B.

Absence of specific DELLA combinations suppresses *ga1-3* floral phenotype

To investigate if DELLA proteins are repressors of floral development, we studied floral development in *ga1-3* plants lacking various combinations of GAI, RGA, RGL1 and RGL2.

First, we studied *ga1-3* plants lacking single DELLA proteins. Absence of RGL1, RGL2 or GAI alone had little obvious effect on the phenotype of *ga1-3*, while absence of RGA alone partially suppressed the stem elongation phenotype of *ga1*-3 (Silverstone et al., 1998) (Fig. 5A). However, absence of RGA alone did not restore normal floral development or fertility to *ga1-3* (Fig. 5C).

Second, we studied *ga1-3* plants lacking all possible pair-wise combinations of RGL1, RGL2, GAI or RGA. Lack of GAI and RGA completely suppressed the dwarf phenotype conferred by *ga1*-3 (Dill and Sun, 2001; King et al., 2001) (Fig. 5B). All other combinations caused a phenotype that was indistinguishable from that of *ga1-3* (plants lacking RGL1 and RGL2, or lacking RGL1 and GAI, or lacking RGL2 and GAI) or from *ga1-3* plants lacking RGA (plants lacking RGL1 and RGA, or lacking RGL2 and RGA). Thus, GAI and RGA in combination play the predominant role in controlling stem growth. Absence of RGL1 and RGL2 did not obviously enhance the suppression of *ga1-3* phenotype because of absence of RGA, suggesting that RGL1 and RGL2 have relatively minor roles in the regulation of stem elongation.

For the most part, the pair-wise DELLA absence combinations failed to confer normal flower development on *ga1-3* (Dill and Sun, 2001; King et al., 2001). All of these lines produced flower buds, but the buds failed to open and exhibited the arrested petal and stamen growth characteristic of *ga1-3* (Fig. 7A). However, some flower opening was observed in the late maturity of two of the pair-wise DELLA absence combination lines. Although flowers of 40-day old *ga1-3* plants lacking RGL2 and RGA were sterile, at ~50 days and older these plants produced flowers that opened and were able to set seed (Fig. 5C). In addition, the flowers of late maturity *ga1-3* plants lacking GAI and RGA (~55 days old; data not shown) sometimes opened, but these flowers were almost always sterile.

RGL1, RGL2 and RGA act synergistically in the repression of Arabidopsis stamen and petal development

We next studied *ga1-3* plants lacking all possible three-way combinations of RGL1, RGL2, GAI and RGA. Absence of RGL1, RGL2 and GAI failed to suppress any detectable aspect of *ga1-3* phenotype (Fig. 6A,B and Fig. 7A). Absence of

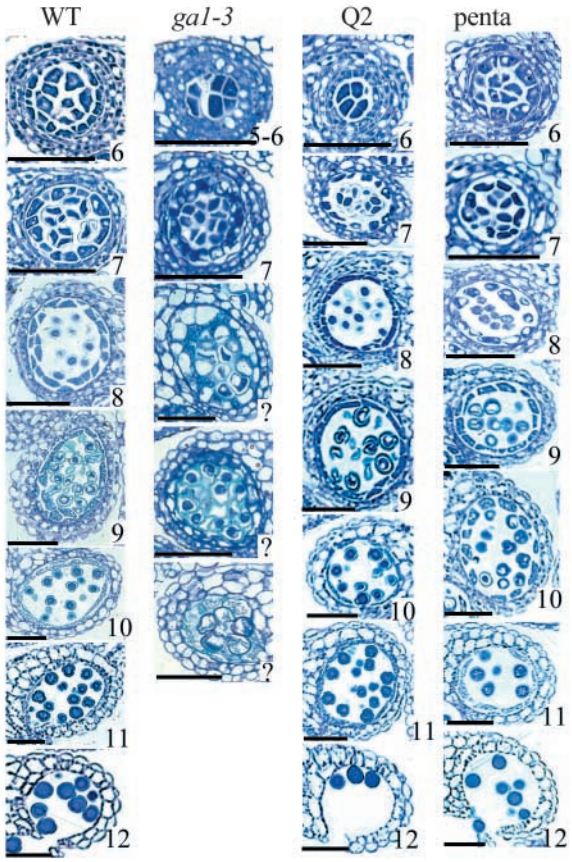


Fig. 3. Histological analysis of microsporogenesis. Transverse sections of anthers are displayed in developmental sequence, showing the progress in microsporogenesis in various genotypes. *ga1-3* anthers developed normally up to the tetrad formation stage (stage 7) but after this, they diverted from the normal (compared with stages 9-12 in wild type; *ga1-3* stages highlighted with question marks). Eventually, all *ga1-3* pollen sacs aborted (last two photos). *ga1-3* plants lacking RGL1, RGL2 and RGA (Q2), or RGL1, RGL2, GAI and RGA (penta) successfully completed microsporogenesis and released mature viable pollen grains. Scale bar: 50 μm. Microsporogenesis stages are indicated in bottom right-hand corner of the pictures.

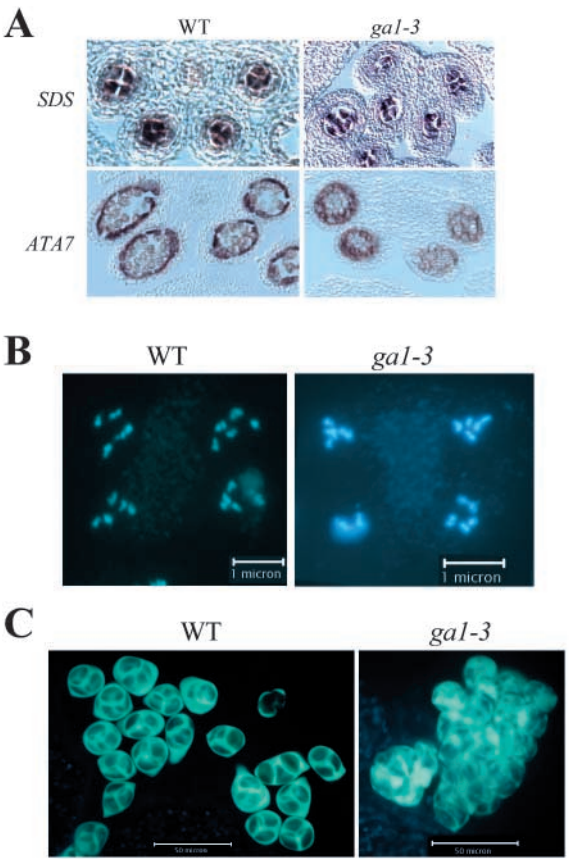


Fig. 4. Pollen development is arrested in *ga1-3*. (A) The anther-specific markers *SDS* and *ATA7* were used in in situ hybridization analysis to compare microsporogenesis in *ga1-3* and wild type. *SDS* patterns appeared the same in *ga1-3* and wild type whereas the *ATA7* signal pattern in *ga1-3* locules was significantly different to that of the wild-type control. (B) Chromosome spread experiments confirmed that pollen meiosis is successfully completed in *ga1-3*, resulting in tetrad formation. (C) Aniline Blue staining showed that the tetrads in *ga1-3* tend to be clustered and are not found in the form of free microspores as is seen in the wild type. Scale bars: 1 μm in B; 50 μm in C.

RGL1, RGL2 and RGA completely restored petal and stamen development to *ga1-3* (Fig. 1A, Fig. 7A), and permitted normal seed set (Fig. 6B, Fig. 7B), despite the fact that this line was semi-dwarf and exhibited a stem elongation phenotype only slightly taller than that of *ga1-3* plants lacking RGA alone (compare Fig. 5A with Fig. 6B). Although *ga1-3* plants lacking RGL2 and RGA produced fertile flowers only in late maturity, *ga1-3* plants lacking RGL1, RGL2 and RGA produced fertile flowers at the onset of flowering. Thus RGL1, RGL2 and RGA act in combination to control petal and stamen development in response to GA.

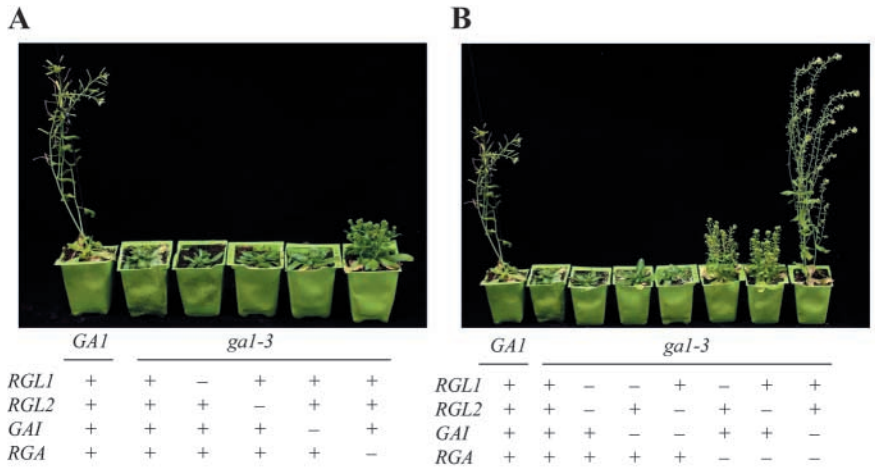
ga1-3 plants lacking RGL1, GAI and RGA were taller than *ga1-3* plants lacking GAI and RGA alone (Fig. 6A,B), suggesting that RGL1 has a significant role in the control of stem elongation when RGA and GAI are absent. However, lack of RGL1, GAI and RGA did not restore normal stamen and petal development to *ga1-3* (Fig. 1B, Fig. 7A), and this line was therefore sterile (Fig. 7B). In fact, the young flower buds of *ga1-3* plants lacking RGL1, GAI and RGA were indistinguishable from those of *ga1-3* plants lacking GAI and RGA only (Fig. 7A).

ga1-3 plants lacking RGL2, GAI and RGA were also taller at maturity than control lines lacking GAI and RGA alone (Fig. 6A,B,D). In contrast to what was seen with lack of RGL1, lack of RGL2 (in *ga1-3* plants lacking RGL2, GAI and RGA) partially restored petal and stamen development to *ga1-3* plants lacking GAI and RGA, making this line partially fertile (Fig. 1B and Fig. 7A,B).

In summary, the results described in this section indicate that GA-regulation of *Arabidopsis* petal and stamen elongation is mediated via RGL1, RGL2 and RGA, with RGL2 and RGA playing the predominant roles.

Absence of RGA, RGL2, RGL1 and GAI leads to GA-independent plant growth

Finally, we analysed *ga1*-3 plants lacking RGL1, RGL2, GAI and RGA. We found that this mutant line bolted and flowered



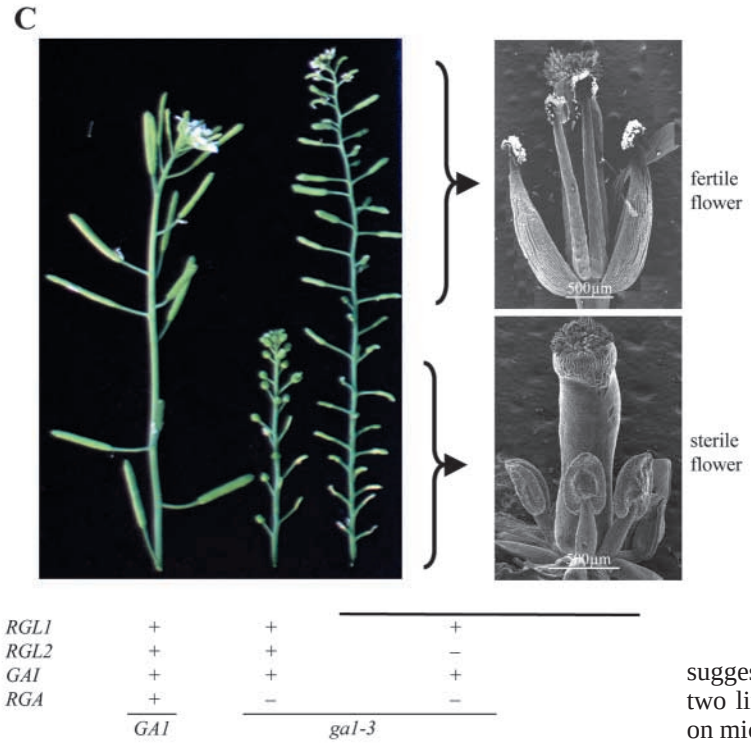


Fig. 5. RGA and RGL2 are key GA-response regulators of floral development. (A,B) Comparison of growth of *ga1-3* plants lacking single (A) or pairs of (B) DELLA proteins at 48 days. Presence of wild-type gene (and functional protein) is indicated by +, presence of loss-of-function mutation (and lack of functional protein) is indicated by –. (C) *ga1-3* plants lacking RGA and RGL2 initially produced sterile non-opening flowers (bottom right; SEM far right), then began to produce fertile open flowers (top right; SEM far right) when they were ~50 days old. By contrast, *ga1-3* plants lacking RGA alone (middle) never produced any fertile flowers.

earlier than wild type both in long (LD) (Fig. 6A,C) and short (SD) days (Fig. 6C). Furthermore, *ga1-3* plants lacking RGL1, RGL2, GAI and RGA were taller than the wild-type control (Fig. 6B,D). In addition, combined absence of RGL1, RGL2, GAI and RGA suppressed the effects of *ga1-3* on petal and stamen development. The flowers of *ga1-3* plants lacking RGL1, RGL2, GAI and RGA exhibited fully extended stamens and petals (Fig. 7A). Anther development proceeded to completion, resulting in flowers that were fertile and set seeds in both LD (Fig. 7B) and SD (data not shown).

We next analyzed stamen filament growth in *ga1-3* plants lacking RGL1, RGL2, GAI and RGA and found that these filaments were slightly longer than those of the wild-type control (Fig. 1A,B). In fact, removing only RGL1, RGL2 and RGA from *ga1-3* also resulted in filament lengths longer than that of wild type (Fig. 1A,B). SEM of stamen filament epidermal cells indicated that restoration of stamen filament length in *ga1-3* mutant plants lacking RGL1, RGL2, GAI and RGA was due to an increase in cell length (Fig. 1C) as opposed to an increase in cell number (Fig. 1D), a difference similar to what was previously observed between wild-type and *ga1-3* stamen filaments. Thus, the elongation of stamen filaments becomes GA independent when all four DELLA proteins are removed.

SEM analysis of pollen grains from *ga1-3* plants lacking RGA, RGL2 and RGL1 showed that their surface structure was indistinguishable from those of wild type (Fig. 2A), being oval-shaped with long indented lines. However, pollen grains from *ga1-3* plants lacking RGL1, RGL2, GAI and RGA were substantially different from those of wild type, mostly having a wrinkled appearance, and sometimes being markedly deformed (Fig. 2A). Although *ga1-3* plants lacking RGL1, RGL2 and GAI had hugely different stem elongation phenotypes to *ga1-3* plants lacking RGL1, GAI and RGA (Fig. 6A,B), the floral phenotypes of these two lines are very similar and both lines had ~30% tricellular pollen (Fig. 2C). This suggests that microsporogenesis is partially restored in these two lines. Lack of both RGL2 and RGA had a greater effect on microsporogenesis, as ~60% or ~80% of pollen grains were found to be tricellular in *ga1-3* plants lacking RGL2, GAI and RGA or RGL1, RGL2 and RGA respectively (Fig. 2B,C). These results indicate that RGA and RGL2 play important roles in the repression of microsporogenesis in *Arabidopsis*, and that GA regulates microsporogenesis by overcoming the repressing effects of RGA and RGL2.

Transverse sectioning showed that *ga1-3* plants lacking RGL1, RGL2 and RGA or *ga1-3* plants lacking RGL1, RGL2, GAI and RGA both achieved complete microsporogenesis (Fig. 3). However, although no obvious differences were observed between wild-type and *ga1-3* plants lacking RGL1, RGL2 and RGA, we often observed that one or two of the four locules of the anthers of *ga1-3* plants lacking RGL1, RGL2, GAI and RGA were aborted (data not shown).

Discussion

DELLA proteins act as repressors of growth whose function is opposed by GA (Richards et al., 2001). In several cases,

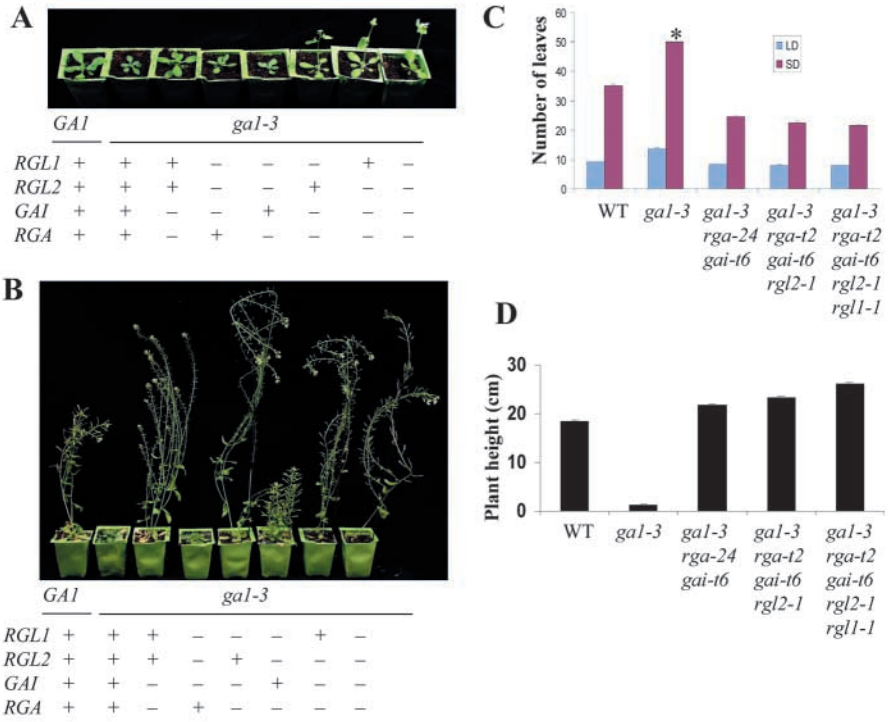


Fig. 6. Absence of RGL1, RGL2, GAI and RGA leads to GA-independent plant growth. (A,B) Wild-type plants were compared with *ga1-3* plants containing loss-of-function mutations causing lack of various combinations of RGL1, RGL2, GAI or RGA at 22 (A) and 48 (B) days. Note that *ga1-3* plants lacking RGL1, GAI and RGA or RGL2, GAI and RGA or all four DELLA proteins bolted earlier than the wild-type control at 22 days. Genotypes are as indicated. (C) Flowering time of various genotypes, measured as number of leaves at time of flowering. In this case, *ga1-3* plants under SD did not produce any visible flower buds even though they had more than 50 leaves (marked with *). Under LD condition, *ga1-3* plants lacking RGL1, GAI and RGA or RGL2, GAI and RGA or all four DELLA proteins all flowered with one or two leaves less than did the wild-type control, although this small magnitude of difference is hard to discern from the histogram shown here. (D) Plant height of various genotypes at 28 days old under LD growth condition. Results are presented as mean±standard error (n=30).

GA opposes DELLA function by promoting DELLA disappearance, most likely via ubiquitin-ligase dependent targetting to the proteasome and subsequent protein degradation (Silverstone et al., 2001; Ito et al., 2002; Gubler et al., 2002; Fu et al., 2002). Recent studies have identified candidate F-box components of a SCF E3 ubiquitin ligase apparently responsible for targetting DELLA proteins to the proteasome (McGinnis et al., 2003; Sasaki et al., 2003). Thus, one emerging picture is that GA stimulates GA-responses by targetting DELLA protein growth repressors for destruction in the proteasome (Harberd, 2003). In another case, that of *RGL2*, GA apparently opposes RGL2 function by causing downregulation of *RGL2* transcript levels during seed germination (Lee et al., 2002).

Previous studies of *Arabidopsis* DELLA function have involved phenotypic comparisons of GA-deficient (*ga1-3*) plants with GA-deficient plants lacking GAI, RGA, RGL1, or RGL2 or a limited range of combinations of these factors (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002). In this paper, we described the effects of a more comprehensive set of DELLA-lack combinations, focussing especially on floral development.

Flowering consists of three distinct phases: floral initiation (in which the vegetative meristem is transformed into an inflorescence meristem), floral organ initiation and floral organ growth. As shown previously, lack of GAI and RGA substantially suppresses the effect of the *ga1-3* mutation on flowering time (a measure of time of floral initiation) in SD (Dill and Sun, 2001). We have shown that an additional lack of RGL2 or of both RGL1 and RGL2 further advances the flowering time (in both LD and SD) of *ga1-3* plants lacking GAI and RGA. However, the magnitude of this further advance is relatively small compared with that initially caused by lack of both GAI and RGA. Thus, GAI and RGA play the predominant role in regulating flowering time in the GA-signalling floral promotive pathway (Simpson and Dean, 2002), with only small contributions from RGL1 and RGL2.

By contrast, RGL1, RGL2 and RGA play key roles in floral organ development. The temporal coordination of the development of individual floral organs is essential for floral function. For example, at around the time that the pollen matures and is dehisced from the anther, the stamen filaments of flowers of self-fertilizing species such as *Arabidopsis* elongate and bring the pollen into contact with the stigmatic papillae (Smyth et al., 1990; Bowman, 1994). We showed that the relatively short stamen filaments of *ga1-3* flowers result from an arrest of cell elongation rather division and that combined lack of RGL1, RGL2, GAI and RGA restored stamen filament cell elongation in *ga1-3* plants. We also showed that, in general, microspores do not proceed to the formation of mature pollen in *ga1-3* anthers, that microspore development is possibly arrested prior to pollen mitosis in *ga1-3*, and that tapetal development is perturbed in *ga1-3*. Whether the effect of *ga1-3* on pollen mitosis is a secondary effect of arrested tapetal development, or is independent of the effect on tapetal development is at present unclear. In addition, we occasionally observed *ga1-3* flower buds containing a significant number of tricellular pollen grains. Further investigation is needed to find out if this is a true reflection of the *ga1-3* developmental process or is caused by other unknown environmental cues. Lack of RGL1, RGL2, GAI and RGA proteins restored microsporogenesis in *ga1-3* plants. Further genetic analysis enabled us to identify RGL2, RGA and RGL1 as the key GA-response regulators controlling stamen filament length and microsporogenesis. Interestingly, pollen grains from *ga1-3* plants lacking GAI, RGL1, RGL2 and RGA, although tricellular and viable, are deformed when compared with the wild-type-appearing pollen grains from

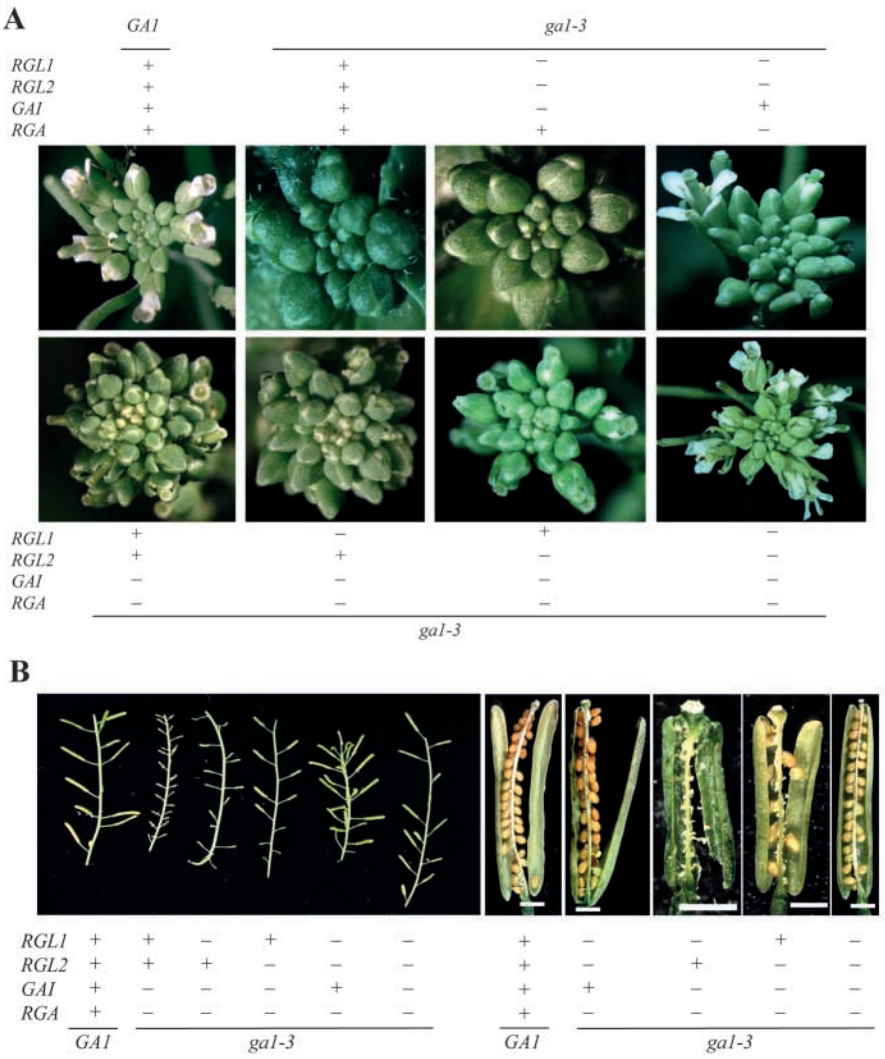


Fig. 7. RGL1, RGL2 and RGA repress flower opening, petal and stamen development in *ga1-3* plants. (A) Comparison of the flowers of 30-day-old plants; genotypes as indicated. (B) Comparison of the seed set of various genotypes. (Left panel) Segment of main shoots bearing siliques. (Right panel) Seed production in a typical silique from genotypes as shown. Although *ga1-3* plants lacking RGL1, GAI and RGA, or RGL2, GAI and RGA both have short siliques, the former bears no seeds whereas the latter is partially fertile. Siliques of *ga1-3* plants lacking RGL1, RGL2 and RGA or lacking all four DELLA proteins are similar not only in length but seed production as well as in wild type. Scale bar: 1.0 mm.

ga1-3 plants lacking RGL1, RGL2 and RGA. Perhaps absence of all four DELLA proteins activates the GA pathway to such high levels that pollen wall materials are overproduced, resulting in abnormal pollen morphology.

Previous developmental genetic analyses showed that the *Arabidopsis* DELLA proteins GAI and RGA act as repressors of stem elongation and that GA exerts its promotive effects on stem growth by overcoming the effects of GAI and RGA (Dill and Sun, 2001; King et al., 2001). These observations, and additional observations on the behaviour of DELLA proteins in other species, have been incorporated into a general 'release of restraint' model, which envisages DELLA proteins as general agents of restraint of plant organ growth, and GA as a means of overcoming that restraint (Peng et al., 1997; King et al., 2001; Richards et al., 2001; Harberd, 2003). However, the initial experiments (which examined the effect of lack of *Arabidopsis* GAI and RGA) showed that although stem elongation could be explained in terms of the 'release of restraint' model, other aspects of growth and development which were known to be GA regulated (in particular seed germination and floral organ growth) could not (Dill and Sun, 2001; King et al., 2001). It therefore remained possible that some other, entirely different, mechanism was responsible for the GA-mediated regulation of seed germination and floral organ growth.

Recently, it has been reported that the GA-promotion of *Arabidopsis* seed germination can be explained in terms of a GA-mediated release of the restraint upon germination imposed by RGL2 (Lee et al., 2002) or RGL1 (Wen and Chang, 2002). The results in this present paper show for the first time that the GA-regulation of floral organ development is also DELLA-mediated. However, different combinations of DELLA proteins are key to floral organ development (RGA, RGL1, RGL2), seed germination (RGL2 and RGL1) and stem elongation (RGA, GAI). The three key aspects of the *ga1-3* mutant phenotype (dwarfism, inhibition of seed germination, retarded floral organ development) can now be explained: the lack of GA in this mutant causes a failure to overcome the repressive effects of the DELLA protein combinations that are specific to each particular phenotypic aspect. As a consequence, the 'release of DELLA restraint' hypothesis can now be considered to be a viable model with which to understand GA responses in general. One possible explanation for how different DELLA combinations control different developmental processes (e.g. seed germination versus stem elongation versus stamen development) is that individual DELLA proteins have different temporal and spatial expression patterns. For example, *GAI* and *RGA* are ubiquitously expressed in all plant tissues, whereas *RGL1* and *RGL2* transcripts are relatively enriched in the inflorescence (Silverstone et al., 1998; Lee et al., 2002; Wen and Chang, 2002). In situ hybridisation showed that *RGL1* is highly expressed in the stamen primordium (Wen and Chang, 2002); however examination of an *RGL2* promoter-GUS fusion line showed that *RGL2* transcripts are also enriched in the stamen (Lee et al., 2002). The expression patterns of *RGL1* and *RGL2* are therefore consistent with our current observation that RGL1 and RGL2 are both important for stamen development. It is possible that RGL3 also plays an important role in various aspects of GA-mediated developmental regulation. Determination of the relative role of RGL3 awaits

characterization of *Arabidopsis* mutants lacking the functional *RGL3* allele.

The nature of the arrest in flower development conferred by *ga1-3* (and restored by lack of RGL1, RGL2 and RGA) is particularly interesting. Our results identify a relatively distinct developmental stage at which arrest occurs. Before that stage, *ga1-3* stamen and anther development proceeds in a way that is indistinguishable from that of wild type. After that stage, wild-type development continues, while *ga1-3* development is blocked. It will be interesting to determine if other GA-deficiency phenotypes (e.g. the particular shape of leaves of *ga1-3* mutant plants) are also due to premature arrest of an identifiable developmental sequence.

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