Repression of AGAMOUS-LIKE 24 is a crucial step in promoting flower development

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Flower development begins as floral meristems arise in succession on the flank of the inflorescence meristem. Floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) promote establishment and maintenance of floral identity in newly formed floral primordia^{1–9}. Without their activity, the floral primordia develop with inflorescence characteristics. The underlying molecular-genetic mechanism is unknown. Here we show that these phenotypes are due in large part to the ectopic expression of *AGAMOUS-LIKE 24* (*AGL24*), a central regulator of floral meristem identity. We present evidence that *AGL24* is an early target of transcriptional repression by *LFY* and *AP1*. Without such repression, continued *AGL24* expression in floral meristems is sufficient to cause floral reversion regardless of the activation of floral organ identity genes. This indicates that *LFY* and *AP1* promote floral development not only by positively regulating genes activated in flower development, but also by repressing *AGL24*, a promoter of inflorescence fate.

The transition from vegetative to reproductive growth is a marked developmental change in the life of plants, whereby newly formed meristems acquire floral rather than inflorescence identity. Inflorescence meristems are indeterminate, with the production of secondary inflorescences subtended by cauline leaves, or flowers, in a spiral pattern, with internode elongation. The floral meristems that originate from inflorescence meristems are determinate, making the four types of floral organs in whorls. The fate of these meristems depends on an antagonistic interaction between the shoot identity gene *TERMINAL FLOWER 1* (*TFL1*) and a group of floral meristem identity genes, such as *LFY* and *AP1* (refs. 6,7,9). Previous studies have shown that *TFL1* acts predominantly to oppose establishment of floral meristem identity but does not antagonize the maintenance of floral meristems^{6,7,10–12}. The simple establishment of floral meristem identity is insufficient to guarantee floral identity, as indicated by the observation that a floral meristem can revert to a flower-generating inflorescence meristem, a phenomenon called floral reversion^{5,13–15}. Floral reversion occurs for the most part in mutant genetic backgrounds, such as *ap1*, *lfy* and *agamous*, suggesting that wild-type plants have default processes to suppress this reversion, and that active maintenance of floral meristem identity is required after its

establishment. It has been proposed that the primary effect of *LFY* in maintaining floral meristem identity may be transcriptional repression⁹, but the potential targets are unknown.

The first indication that *AGL24*, a member of the MADS-box family of DNA-binding transcription factors, is involved in floral meristem identity came from the phenotype of constitutive expression of *AGL24*. Such expression changes flowering time^{16,17} and causes floral reversion. *35S:AGL24* transgenic lines have a uniform, dominant and heritable alteration in flower development. The most notable aspect of this alteration is the partial reversion of floral meristems into inflorescence shoots, which was reminiscent of the effect of strong *ap1* mutated alleles (Fig. 1a). In a homozygous *35S:AGL24* line, the floral meristem that would develop into a single flower in a wild-type plant often generated a central primary flower with extra secondary flowers in the axils of sepals (Fig. 1b). At later stages, the base of the ovary in a *35S:AGL24* flower elongated as an inflorescence stem would, and an ectopic inflorescence bearing flowers erupted from the developing ovary (Fig. 1d,e). Furthermore, most of the floral organs in *35S:AGL24* flowers underwent homeotic transformations toward structures normally found in inflorescence shoots (Fig. 1f–i). These phenotypes were attenuated in *35S:AGL24* hemizygotes (Fig. 1c). The observed homeotic phenotypes of *35S:AGL24* plants were not due to the cosuppression of other MADS-box family members, because both expression levels and patterns of floral organ identity genes were almost identical in *35S:AGL24* and *agl24* plants (Supplementary Fig. 1 online).

The phenotype of *35S:AGL24* plants suggests that *AGL24* promotes inflorescence identity. To test whether the increased inflorescence character of the *lfy* and *ap1* mutants resulted from ectopic expression of *AGL24*, we crossed the loss-of-function mutation *agl24* into the lines with reduced *LFY* and *AP1* activity. The inflorescence phenotype of *ap1-1* mutants was the appearance of ectopic flowers or inflorescences in the axils of the leaf-like first whorl floral organs, creating a supernumerary inflorescence apex (Fig. 2a,c). Floral meristems in *ap1-1* showed some of the same characteristics as inflorescence meristems (Fig. 2e), to a degree that varied with their position on the main inflorescence stem (Supplementary Table 1 online). Supernumerary inflorescences were markedly suppressed in *ap1-1 agl24* double mutants relative to *ap1-1* single

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Figure 1 Phenotypes of constitutive expression of *AGL24*. (a) *ap1-1* flower had bract-like sepals and secondary flowers (arrows) without petals. (b) *35S:AGL24* flower had similar phenotypes as *ap1-1* (a). Note the formation of a secondary flower (arrow). (c) *35S:AGL24/-* flower had the attenuated phenotype with only bract-like sepals. (d) Ectopic inflorescence formation in the swollen ovary of a *35S:AGL24* flower. (e) Development of ectopic inflorescence shown in d, with some carpelloid structures at the margin of cauline leaves (arrow). (f) Scanning electron micrograph (SEM) of a *35S:AGL24* flower shows stellate trichomes on the abaxial surface of the first whorl organs and the homeotic transformation of a petal into a stamen (arrow). (g–i) SEMs of the abaxial surface of a sepal (g), petal (h) and carpel (i) in a wild-type flower. (j–l) SEMs of the abaxial surface of the first whorl (j), second whorl (k) and fourth whorl (l) organs in a *35S:AGL24* flower. Scale bars: a–f, 1 mm; g–l, 50 μ m.

mutants (Fig. 2b,d). Most of the floral meristems in the double mutant developed as single flowers (Fig. 2f and Supplementary Table 1 online). In general, the average number of flowers produced in each pedicel or peduncle of *ap1-1 agl24* mutants was comparable to that produced in wild-type plants. Some floral organ defects of *ap1-1* mutants, especially the absence of petals, were also partly rescued by *agl24* (Fig. 2f–h).

Loss of *AGL24* function also reduced the inflorescence characteristics of *lfy-6* mutants⁴ in three ways. First, flowers in a *lfy-6* single mutant were typically subtended by bracts (Fig. 3a,c,e), as secondary inflorescence meristems in wild-type plants are. In *lfy-6 agl24* double mutants, most flowers developed as in the wild type, without bracts (Fig. 3b,d,f). Second, floral organs were arranged in a spiral pattern in *lfy-6* single mutants (Fig. 3e), as leaves around inflorescence meristems in wild-type plants are. But *lfy-6 agl24*

double mutants had floral organs largely organized in a whorled pattern (Fig. 3f). Finally, *lfy-6* single mutants could have ectopic secondary flowers, but these were not seen in *lfy-6 agl24* double mutants. Overall, the inflorescence architecture of a *lfy-6 agl24* double mutant more closely resembled that of a wild-type plant than that of a *lfy-6* mutant (Fig. 3g). The floral organ defects in *lfy-6* mutants were not rescued in the *lfy-6 agl24* double mutant, which indicates that *LFY* and *AP1* function through distinct mechanisms in the regulation of floral organ identity. *lfy-6 ap1-1* double mutants showed enhanced inflorescence phenotypes as compared with single mutants⁴, and these were rescued in an *agl24* background, as in the single mutants (data not shown). These genetic data indicate that *AGL24* contributes much of the inflorescence character of *lfy* and *ap1* mutants, supporting the possibility that *LFY* and *AP1* act to repress *AGL24* in wild-type plants.

To test directly the potential repression of *AGL24* by the floral meristem identity genes, we carried out *in situ* hybridization with the *AGL24* probe to *lfy-6* and *ap1-1* inflorescences. In wild-type plants during their vegetative phase, *AGL24* is expressed in the entire shoot apical meristem and in emerging leaf primordia¹⁶. During floral transition, *AGL24* RNA was detectable in the transitional shoot apex and in young cauline leaves (Fig. 4d). *AGL24* expression in early floral meristems differed from that in inflorescence meristems. In floral meristems from stages 1 to 4, *AGL24* was expressed only in the tunica, the superficial layers of the meristems (Fig. 4a). The absence of *AGL24* RNA in the corpus of floral primordia coincided with the developmental stages at which *LFY* and *AP1* are expressed in the floral primordia^{4,18}. Thus, in the corpus but not in the tunica, the activity of *LFY* and *AP1* may repress *AGL24*. At later stages of floral development, *AGL24* RNA was weakly expressed in the distal parts of stamens and carpels (Fig. 4b). Its expression was restricted to pollen and the adaxial surface of the gynoecium after floral stage 9 (Fig. 4c).

Figure 2 Rescue of *ap1-1* by *agl24*. Light micrographs of *ap1-1* (a,c,e,g) and *ap1-1 agl24* (b,d,f,h) mutants are shown. (a,b) Top view of a developing inflorescence. (c,d) Side view of a developing inflorescence. (e,f) Side view of a floral structure arising from an individual floral meristem at a basal position in the main inflorescence. (g,h) Side view of a flower arising at an apical position in the main inflorescence.

Figure 3 Rescue of *lfy-6* by *agl24*. Phenotypes of *lfy-6* (a,c,e) and *lfy-6 agl24* (b,d,f) mutants are shown. (a,b) Side view of a developing inflorescence. (c,d) Top view of a developing inflorescence. The cauline leaf-like bracts subtending flowers in *lfy-6* mutants (a,c) were lost in *lfy-6 agl24* mutants (b,d). (e,f) SEM of an inflorescence apex. Bract structures (arrows) surrounding floral meristems in *lfy-6* mutants (e) disappeared in *lfy-6 agl24* mutants (f), and the spiral mode of floral organ formation (arrowhead) in *lfy-6* mutants became the whorled mode (arrowhead). (g) Schematic representation of the rescue of *lfy-6* by *agl24*. The mean numbers of different structures calculated from 20 samples are given. The *lfy-6 agl24* double mutant produced around eight secondary inflorescences, four flowers subtended by bracts, as in *lfy-6* mutants, and the rest of the apical flowers without bracts, as in a wild-type plant. Scale bars: e,f, 100 μ m.

In *ap1-1* mutants, *AGL24* was ectopically expressed in the corpus of the young floral primordia (Fig. 4e), and its strong expression was persistent in all of the floral organs at later stages of flower development (Fig. 4f,g). We observed similar ectopic expression of *AGL24* in the supernumerary meristems of *ap1-1 cal-1* mutants (Fig. 4h), indicating that ectopic expression of *AGL24* was responsible for the enhanced inflorescence traits in *ap1-1 cal-1* mutants. In *lfy-6* mutants, ectopic expression of *AGL24* was also apparent throughout the young floral meristems (Fig. 4i), which coincided with the reduced expression of *API* RNA in the same region (Fig. 4m,n). The later expression of *API* in *lfy-6* mutants is comparable to that in the wild type (Fig. 4o,p). The ectopic expression of *AGL24* in emerging floral primordia and the corresponding cauline leaves in an *lfy-6* mutant (Fig. 4l) seemed stronger than its expression in the transitional inflorescence meristem of a wild-type plant (Fig. 4d). This indicates that floral primordia in *lfy-6*

mutants initiated with some degree of inflorescence character. *AGL24* RNA in *lfy-6* mutants was also distributed in floral organ primordia (Fig. 4j), predominantly in the irregularly fused carpels at later stages (Fig. 4k). It is therefore possible to conclude that the increase of inflorescence traits in flowers of *ap1-1* and *lfy-6* mutants, which mimics the phenotypes of gain of function of *AGL24*, resulted from ectopic expression of *AGL24*.

To further confirm that *LFY* and *API* act as transcriptional repressors of *AGL24*, we used steroid-inducible versions of *API* and *LFY* in transgenic plants¹⁹. Before using the *API-GR* construct, we tested its biological activity. The development of 35S:*API-GR* transformants was indistinguishable from that of wild-type plants in the absence of applied steroid. After treatment with dexamethasone, both the primary and secondary inflorescences of 35S:*API-GR* plants terminated as floral meristems and formed terminal flowers, which is characteristic of *API*

Figure 4 *In situ* localization of *AGL24* (a–l) and *API* (m–p) RNA. (a–d) *AGL24* expression in wild-type plants. *AGL24* was expressed in the inflorescence meristem and the tunica region of young floral meristems (a and inset), in the distal region of stamen and carpel primordia (b) and later in the pollen and the adaxial surface of the gynoecium (c). During the floral transition, *AGL24* RNA was distributed in the shoot apex and the corresponding cauline leaves (d). (e–g) *AGL24* expression in *ap1-1*. *AGL24* was ectopically expressed in the whole zone of floral meristems (e) as well as the stamen and carpel primordia (f), and later in the petals, anthers and gynoecium (g). (h) Ectopic *AGL24* expression in the supernumerary meristems in *ap1-1 cal-1*. (i–l) *AGL24* expression in *lfy-6*. *AGL24* was ectopically expressed in the whole zone of floral meristems (i) and several organ primordia (j), and later mainly confined to the central region of the flower (k). *AGL24* expression was stronger in the young floral primordium and the coupled cauline leaf-like bract (l) than in the transitional inflorescence apex (d). (m–p) *API* expression in *lfy-6* (m,o) and wild-type plants (n,p). In *lfy-6*, the initial expression of *API* was much lower (m) than in the wild-type plant, and its later expression (o) was comparable with that in the wild-type plant (p). Scale bars, 100 μ m.

Figure 5 Transcriptional repression of *AGL24* by *AP1* and *LFY*. (**a–d**) A biologically active *AP1-GR* fusion. Individual seedlings of 7-day-old *ap1-1* 35S:*AP1-GR* plants were mock-treated (0.03% ethanol and 0.015% Silwet L-77) or treated with 10 μ M dexamethasone and 0.015% Silwet L-77 twice with a one-week interval between treatments. Sixteen days after the first treatment, mock-treated plants had the same inflorescences as *ap1-1* mutants (**a**), whereas dexamethasone-treated plants had a rescued phenotype with terminal flowers (**b**), mimicking 35S:*AP1* plants. The rescue of floral organ defects, such as the generation of sepals and petals, was obvious in late-stage inflorescences of dexamethasone-treated *ap1-1* 35S:*AP1-GR* plants (**c**), in which most flowers were fully rescued (**d**). (**e**) Time-course expression of *AGL24* in inflorescences of *ap1-1* 35S:*AP1-GR* and *lfy-6* 35S:*LFY-GR* plants mock-treated (M) or treated with 10 μ M dexamethasone (D). (**f**) Expression of *AGL24* and *AP1* in inflorescences of *lfy-6* 35S:*LFY-GR* plants, and expression of *AGL24* in inflorescences of *ap1-1* 35S:*AP1-GR* plants. The inflorescences were mock-treated (M) or treated with 10 μ M dexamethasone (D), with 10 μ M cycloheximide (C) or with 10 μ M cycloheximide plus dexamethasone (CD). Expression analyses were done after 4 h of treatment. In (**e**) and (**f**), the numbers below each lane indicate the relative expression levels of each studied gene, calculated by normalizing each signal first against the signal for *TUB2* and then against the first-lane value.

gain-of-function plants²⁰. Activation of 35S:*AP1-GR* in an *ap1-1* genetic background resulted in partial or full rescue of *ap1-1* mutant phenotypes (**Fig. 5a–d**), suggesting that the *AP1-GR* fusion was biologically functional in a hormone-dependent manner.

Treatment with dexamethasone of inflorescence apices of *ap1-1* 35S:*AP1-GR* plants for 4 h or longer resulted in a reduction of *AGL24* RNA level relative to mock-treated controls (**Fig. 5e**). A combined treatment of dexamethasone and cycloheximide, an inhibitor of translation, at the 4-h time point resulted in a similar reduction of *AGL24* RNA as observed with dexamethasone treatment alone (**Fig. 5f**), indicating that *AGL24* may be an immediate target of transcriptional repression by *AP1*. We also investigated *AGL24* expression in the established 35S:*LFY-GR* system¹⁹. *AGL24* RNA level was decreased after treatment of *lfy-6* 35S:*LFY-GR* plants

with dexamethasone for 4 h or more (**Fig. 5e**), suggesting that *LFY* also acts as a repressor of *AGL24*. But we did not observe similar repression after a combined treatment with dexamethasone and cycloheximide (**Fig. 5f**), indicating that this repression may be modulated by other mediators that require translation after *LFY* activation, which may include *AP1*.

Taken together, the results presented here suggest that *AGL24* is among the central regulators of floral meristem development, acting to promote inflorescence identity, and is repressed by the floral meristem identity genes *LFY* and *AP1*. This negative interaction identifies two antagonistic genetic pathways in flower development (**Fig. 6**), in which *LFY* and *AP1* not only activate the ABC floral organ identity genes to promote flower development^{21–23}, but also repress *AGL24* to secure the maintenance of floral rather than inflorescence meristem identity.

METHODS

Plant materials. All mutations used in this study are in the Landsberg *erecta* (*Ler*) background except when stated otherwise. We grew them at 22 °C in continuous light. We obtained the *Ler* near-isogenic *agl24* line by three backcrosses of the *agl24* Columbia line into *Ler*¹⁶. We identified the *lfy-6 agl24* and *ap1-1 agl24* double mutants by screening for mutant phenotypes and confirmed their identification by PCR genotyping of all three genes. We genotyped *agl24*, *lfy-6*, and *ap1-1* as reported previously^{8,17,24}. We screened 35S:*AP1-GR* plants by Basta selection and further tested them for phenotypic effects after dexamethasone treatment. We selected one transgenic line, which contains only one transgene insertion and shows the phenotype closely resembling 35S:*AP1* transformants²⁰ after hormone induction, to cross with *ap1-1* to generate *ap1-1* 35S:*AP1-GR* plants. The selection of 35S:*AGL24* plants has been described elsewhere¹⁶.

Plasmid constructs. The construction of 35S:*AGL24* is described previously¹⁶. For 35S:*AP1-GR*, we constructed a derivative pGreen-35S vector by cloning a 35S promoter cassette from p35S-2 into the *EcoRV* and *StuI* sites of pGREEN0229 (ref. 25). We amplified the hormone-binding domain of the rat glucocorticoid receptor (GR) by PCR from pBI-AGR²⁶. We removed an internal *EcoRI* site in this GR fragment by silent mutagenesis and cloned the resulting fragment into pBSII-SK (Stratagene). We released the GR fragment from pBSII-SK at the *SacI* and *EcoRI* sites and cloned it into pGREEN0229-35S to generate pGREEN0229-35S:GR. We amplified the *AP1* coding region¹⁸ by PCR and cloned it into the vector pZERO blunt II TOPO (Invitrogen). We then cut

Figure 6 Model for floral meristem development. *LFY* and *AP1* antagonize *TFL1* activity to establish floral meristem (FM) identity on the flanks of the inflorescence meristem (IM) and repress *AGL24* to prevent its interference with the normal functions of floral homeotic genes (FHG). *AGL24* expression is shown in the background, where the putative border between the inflorescence meristem and the floral meristem is indicated as a dotted line.

out the *API* fragment with *Xba*I and *Bam*HI and inserted it into pGREEN0229-35S:GR to yield pGREEN0229-35S:API-GR. We sequenced the construct to detect potential PCR-introduced mutations.

Expression analyses. Dexamethasone treatment and sample collection were previously described¹⁹. We extracted total RNA by RNeasy Plant Mini Kit (Qiagen) and reverse-transcribed it using the ThermoScript RT-PCR system (Invitrogen). We carried out RT-PCR and then detected expression by Southern-blot analysis as described previously¹⁶. We synthesized antisense RNA probes for *in situ* hybridization from plasmids pHY301(AGL24)¹⁶, pDW124(LFY)⁴, pAM128(*API*)¹⁸, pD793(AP3)²⁷ and pCIT565(AG)²⁸. We carried out nonradioactive *in situ* hybridization according to a published protocol²⁹.

Scanning electron microscopy. We prepared samples as described³⁰.

Note: Supplementary Information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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