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AGL24, *SHORT VEGETATIVE PHASE*, and *APETALA1* Redundantly Control *AGAMOUS* during Early Stages of Flower Development in *Arabidopsis* [W]

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Loss-of-function alleles of *AGAMOUS-LIKE24* (*AGL24*) and *SHORT VEGETATIVE PHASE* (*SVP*) revealed that these two similar MADS box genes have opposite functions in controlling the floral transition in *Arabidopsis thaliana*, with *AGL24* functioning as a promoter and *SVP* as a repressor. *AGL24* promotes inflorescence identity, and its expression is downregulated by *APETALA1* (*AP1*) and *LEAFY* to establish floral meristem identity. Here, we combine the two mutants to generate the *agl24 svp* double mutant. Analysis of flowering time revealed that *svp* is epistatic to *agl24*. Furthermore, when grown at 30°C, the double mutant was severely affected in flower development. All four floral whorls showed homeotic conversions due to ectopic expression of class B and C organ identity genes. The observed phenotypes remarkably resembled the *leunig* (*lug*) and *seuss* (*seu*) mutants. Protein interaction studies showed that dimers composed of AP1-AGL24 and AP1-SVP interact with the LUG-SEU corepressor complex. We provide genetic evidence for the role of AP1 in these interactions by showing that the floral phenotype in the *ap1 agl24 svp* triple mutant is significantly enhanced. Our data suggest that MADS box proteins are involved in the recruitment of the SEU-LUG repressor complex for the regulation of *AGAMOUS*.

INTRODUCTION

The transition from vegetative to reproductive growth is a major developmental switch in the life cycle of plants since it is the key step for the reproductive success of the plant. During the vegetative phase, the shoot apical meristem produces leaf primordia. After perception and processing of several environmental and internal signals, the shoot apical meristem undergoes a change in fate, and an inflorescence meristem is produced. This process is called the floral transition (for reviews, see Komeda, 2004; Putterill et al., 2004; He and Amasino, 2005).

The genetic network controlling the floral transition culminates in the activation of floral meristem identity genes, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*), which subsequently regulate the three classes of floral homeotic genes, A, B, and C (Weigel and Meyerowitz, 1994; Komeda, 2004). These ABC floral homeotic genes function in overlapping domains to specify different floral organ identities. For instance, *LFY* positively regulates *AGAMOUS* (*AG*), the class C gene that is responsible for stamen and carpel identities (Bowman et al., 1991), by binding to sites within the *AG* control region that is located within the second intron (Busch et al., 1999; Lohmann et al., 2001). *LFY*, together with *AP1*, is also required for the activation of the class B gene *AP3*, which together with *PISTILLATA* specifies petal and stamen identities (Ng and Yanofsky, 2001; Lamb et al., 2002). *AG* is regulated by several other factors that all seem to act on the second intron (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000; Hong et al., 2003; Bao et al., 2004). One of these factors is *LEUNIG* (*LUG*), which also regulates the expression of class A and B genes (Liu and Meyerowitz, 1995). *LUG* encodes a protein with homology to the Tup1 corepressor from yeast and Groucho from *Drosophila melanogaster* (Conner and Liu, 2000). LUG interacts with SEUSS (SEU) to regulate *AG* (Sridhar et al., 2004). In contrast with LUG, SEU does not exhibit any direct repressor activity. It is a plant-specific protein that shows sequence similarity to the dimerization domain of the LIM domain binding (Ldb) family of transcriptional coregulators, such as Ldb1 in mouse and Chip in *Drosophila* (Franks et al., 2002). By analogy with the Ssn6-Tup1 complex (Conlan et al., 1999), it has been suggested that SEU acts as adapter protein between LUG and DNA binding transcription factors (Sridhar et al., 2004).

Here, we report the analysis of two MADS box genes, namely, *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-LIKE24* (*AGL24*), originally isolated and characterized as regulators of the floral transition (Hartmann et al., 2000; Yu et al., 2002; Michaels et al., 2003). Although phylogenetic analysis showed that these genes are closely related (Pařenicová et al., 2003), functional analyses revealed that they have an opposite effect in the control of flowering time. Whereas *SVP* acts as a repressor of flowering, *AGL24* functions as a promoter of flowering. Both genes are expressed in vegetative tissue before the floral transition. *SVP*

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exerts its repressive function in a dosage-dependent manner, independently from environmental factors such as daylength or temperature (Hartmann et al., 2000). *AGL24* is a dosage-dependent flowering promoter and is gradually activated in the shoot apical meristem during the floral transition (Yu et al., 2002; Michaels et al., 2003). It is one of the key genes in promoting inflorescence fate (Yu et al., 2004), and the meristem identity genes, in particular, *AP1* and *LFY*, downregulate the expression of *AGL24* to establish floral meristem identity and subsequently upregulate the ABC floral organ identity genes (Yu et al., 2004).

svp and *agl24* single mutants are affected only in the transition to flowering. However, after the transition, their expression has also been detected in the inflorescence meristem and during early stages of flower development (Hartmann et al., 2000; Yu et al., 2002, 2004). This overlap in expression pattern combined with a high level of sequence similarity suggests a possible redundant role after the floral transition. Therefore, we combined these two mutants and studied the effects on flowering time and flower development. The obtained phenotypes showed that for the control of flowering time, *svp* is epistatic to *agl24*. Furthermore, flower development was severely affected in this double mutant when grown at 30°C. Both the number and the identity of the floral organs were affected in all floral whorls. In situ expression analysis showed that the homeotic transformations observed in these mutant flowers are possibly due to the deregulation of class B and C identity genes. The observed floral phenotypes are very similar to what has been reported for the *lug* and *seu* mutants. Yeast two-, three-, and four-hybrid protein interaction studies showed that a dimer composed of AP1 and SVP, or AP1 and AGL24 can bind the LUG-SEU corepressor. The role of AP1 in these interactions was further supported by genetic experiments that showed that the floral phenotypes in the *ap1 agl24 svp* triple mutant are significantly enhanced in respect to the *agl24 svp* double mutant. Our data suggest a role for MADS box factors in the recruitment of the SEU-LUG corepressor complex for the regulation of *AG* expression during early stages of flower development.

RESULTS

Flowering Time in the *agl24 svp* Double Mutant

AGL24 and SVP are two MADS box transcription factors that show significant similarity in primary amino acid sequence (identity is 53%, and similarity is 71%). Despite this similarity, they have opposite functions in the transition to flowering. Mutations in *AGL24* confer a dosage-dependent late-flowering phenotype, indicating that *AGL24* is a promoter of the floral transition (Yu et al., 2002; Michaels et al., 2003; Table 1). *svp* mutants show a dosage-dependent early-flowering phenotype, indicating that *SVP* is a repressor of the floral transition (Hartmann et al., 2000). To investigate whether *AGL24* and *SVP* act in the same pathway, an *agl24-2 svp-41* double mutant was created. This double mutant was compared with wild-type and single mutants for differences in flowering time under short-day (SD) (8 h light/16 h dark) conditions. As shown in Figure 1, the *svp-41* mutant is early flowering, forming ~14.6 ± 1 rosette leaves in SD conditions. The *agl24* mutant is late flowering in respect to the wild type, forming on average 75.0 ± 2.5 rosette leaves in SD conditions. These values are comparable to previously published data (Hartmann et al., 2000; Yu et al., 2002; Michaels et al., 2003). The *agl24-2 svp-41* double mutant has the same flowering time as the *svp-41* single mutant (14.5 ± 1 rosette leaves in SD conditions) showing that *svp* is epistatic to *agl24*.

Table 1. Effects of *svp*, *agl24*, *ap1*, *lug*, and *seu* Mutants on Flower Development

Mutant Combinations	Phenotypes	Reference
svp-41	Severe early flowering and normal floral development.	Hartmann et al. (2000)
agl24-2	Late flowering and normal floral development.	Michaels et al. (2003)
agl24 svp	Severe early flowering, mild floral defects at 20°C: reduced number of organs, partial homeotic transformation in whorl 1. Strong floral defects at 30°C: reduced number of organs, homeotic transformation in all whorls due to the deregulation of *AG* and *AP3* expression in floral meristem and organ primordia.	This study
ap1-12	Weak allele, homeotic conversion of sepals into leaves or leaf-like sepals. Few secondary or tertiary flowers are formed in the axil of transformed sepals, reduced number of petals.	G. Haughn[a]
agl24 svp ap1-12	Strong floral defects at 22°C: homeotic conversion of sepals into staminoid or carpelloid organs, petals completely missing, resemble *lug-1* mutant flowers.	This study
lug-1	Narrow leaves and floral organs, homeotic transformations in whorls 1 and 2, reduction in organ number, deregulation of *AG* and *AP3* expression in floral meristem and floral organ primordia.	Liu and Meyerowitz (1995)
seu-1	Floral phenotype similar to, but weaker than, *lug-1*, including narrow leaves and floral organs, homeotic transformations in whorls 1 and 2, organ number reduced in whorls 2 and 3.	Franks et al. (2002)
lug seu	Enhanced flower phenotype compared with both single mutant plants, reduction in floral organ number and enhanced carpelloidy of whorl 1 organs, whorl 2 organs are completely absent. Deregulation of *AG* and *AP3* expression in floral meristem and floral organ primordia.	Franks et al. (2002)

[a] http://nasc.life.nott.ac.uk/.

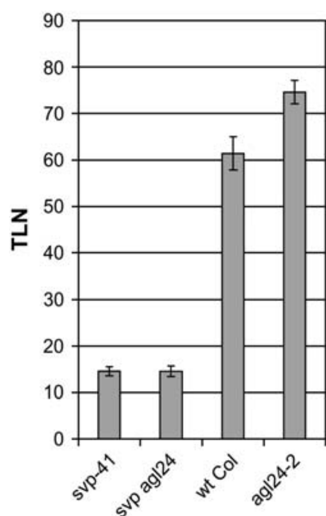

Figure 1. Flowering Time in the *agl24* and *svp* Single and *agl24 svp* Double Mutants.

Flowering time is expressed as the number of rosette leaves formed prior to bolting. The plants were grown under SD conditions. Error bars represent the standard deviation. TLN, total leaf number.

Flower Development in the *agl24 svp* Double Mutant

Although the *svp* and *agl24* single mutants are only affected in flowering time (Table 1), previously reported expression analyses showed that *AGL24* and *SVP* are also expressed after the floral transition in generative tissues (Hartmann et al., 2000; Yu et al., 2002, 2004; Michaels et al., 2003). These experiments showed that *SVP* is expressed in the secondary inflorescence meristems but was absent in the primary inflorescence meristem. *SVP* expression is also detected in the floral primordia until stage 3 when sepal primordia become visible. *AGL24* expression in generative tissue is similar to the *SVP* expression profile. *AGL24* overlaps with *SVP* expression in the secondary inflorescence meristems, although *AGL24* mRNAs were also detected in the primary inflorescence meristem. *AGL24* is also expressed in the floral meristem, and in later stages of flower development, weak expression is observed in stamens and carpels.

Since there is an overlap in expression in generative tissues, we analyzed the *svp agl24* double mutant for defects in flower development (Table 1). Under standard growing conditions (22°C), only the first three flowers of the double mutant were affected. These flowers had a reduced number of organs. In particular, this reduction concerned the second and third floral whorls that produced three petals and five stamens, whereas wild-type *Arabidopsis thaliana* flowers have four petals and six stamens (Figures 2A and 2B). Furthermore, some flowers showed homeotic conversion of sepals into petaloid organs (Figures 2C and 2D).

When double mutant plants were grown at a higher temperature (>30°C), almost all flowers (90%) were severely affected in contrast with wild-type and single mutant plants that showed no floral phenotype. The double mutant flowers exhibited variable floral defects, and all produced a reduced number of organs (Figures 2E to 2I).

Frequently (70% of double mutant flowers) sepals were fused and they showed carpelloid features, including stigmatic papillae and multiple ovules developed along the margin of each organ (Figures 2E and 2H). Less frequently we also observed the homeotic conversion of sepals into petaloid (Figure 2F) or staminoid organs (Figures 2F, 2G, and 2I). In the second whorl, the petals were reduced in number or were completely missing (Figures 2E to 2I). Some plants developed staminoid tissue on top of the petals or stamen-like filaments at the base (Figure 2F). In the third whorl, all flowers had fewer stamens than wild-type flowers, and sometimes they were malformed (Figures 2E to 2I). In the fourth whorl, we sometimes observed defects in carpel fusion (Figures 2E, 2F, and 2H). Furthermore, in some cases, anther tissue developed on top of these unfused carpels (Figure 2E). A few plants (5 out of the 98 that we analyzed) developed terminal flowers, transforming primary or secondary inflorescence meristems into floral meristems. These terminal flowers were in general composed of only carpels and stamens (Figure 2I).

Scanning electron microscopy analysis of young floral buds (stage 6) of wild-type and *agl24 svp* double mutant flowers at 30°C confirmed that development is already affected at early stages in the double mutant flower (Figures 3A and 3B). Wild-type stage 6 flowers are enclosed by sepals, whereas the outer whorl organs of mutant flowers are not covering the inner whorls. In the mutant flower shown in Figure 3B, only two sepal primordia and three normal stamen primordia are formed. Furthermore, one stamen primordia is fused with the gynoecium primordia.

Expression Analysis of Class B and C Genes

The phenotypes described above suggest that in the *agl24 svp* double mutant, homeotic class B and C genes are deregulated, resulting in their ectopic expression. Therefore, we analyzed by in situ hybridization the expression of *AG* and *AP3* during different stages of *Arabidopsis* flower development (Figure 4). In wild-type flowers, *AP3* expression becomes visible in the floral meristem prior to petal and stamen primordia development (stage 3 flowers), and expression is maintained in petals and stamens during all stages of their development (Figure 4B; Jack et al., 1992). The in situ analysis shows that in the *agl24 svp* double mutant, *AP3* is expressed (starting from stage 3) in all parts of the floral meristem. Subsequently, *AP3* expression was detected in all floral primordia and later in all floral organs (Figure 4D).

AG is the class C gene of *Arabidopsis* and is expressed in the inner part of the floral meristem where stamen and carpel primordia develop (Bowman et al., 1989; Yanofsky et al., 1990; Drews et al., 1991). During flower development, *AG* expression is restricted to whorls 3 and 4 (Figure 4A). In the *agl24 svp* double mutant, *AG* mRNAs were already detected in the inflorescence and floral meristems starting from stage 1, indicating precautious *AG* expression. In later stages, *AG* remains expressed in all floral organs.

This expression analysis showed that in the double mutant, *AG* is expressed earlier during flower development, and both *AG* and *AP3* are not restricted to specific floral whorls. Furthermore, the expression of these genes is often less uniform than observed in wild-type whorl 2 and 3 organs, since often we see expression concentrated in patches (Figures 4E and 4F). This misexpression

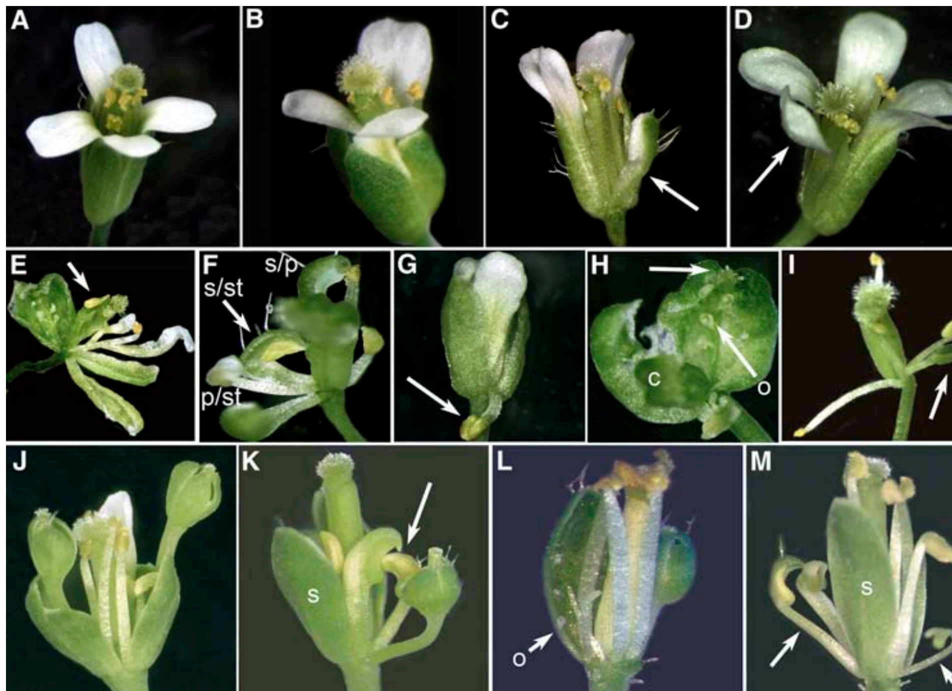

Figure 2. Floral Defects in *agl24 svp* Double and *agl24 svp ap1* Triple Mutants.

(A) Wild-type flower at 30°C.
(B) to **(D)** Flowers of the *agl24 svp* double mutant at 22°C.
(B) Flower with three petals and five stamens.
(C) Flower with three petals; the arrow indicates a petaloid sepal.
(D) Flower with a sepal almost completely converted into a petal; the arrow indicates a petaloid sepal.
(E) to **(I)** Flowers of the *agl24 svp* double mutant at 30°C.
(E) Flower with whorl 1 organs homeotically converted into fused carpelloid organs. The arrow indicates a chimeric organ composed of carpelloid and antheroid tissues.
(F) Flower with chimeric organs in the first, second, and third whorls. Number of organs in these whorls is significantly reduced. p/st, staminoid petal; s/st, staminoid sepal; s/p, petaloid sepal.
(G) Flower having a staminoid organ instead of a sepal (arrow).
(H) Flower with sepals converted into fused carpelloid structures with stigmatic papillae and multiple ovules (o). Whorl 2 and 3 organs are absent. Whorl 4 carpels are not fused, exposing the ovules (c).
(I) Flower having few organs and a chimeric sepal composed of an anther filament and sepal-like tissue (arrow).
(J) *ap1-12* mutant flower with four normal sepals; few secondary flowers are formed in the axils of sepals and reduced number of petals and stamens.
(K) to **(M)** Flowers of the *agl24 svp ap1* triple mutant at 22°C.
(K) Occasionally, secondary flowers are formed in the first whorl. Medial whorl 1 sepals are staminoid (arrow), whereas lateral whorl 1 sepals (s) are normal. Whorl 2 petals are chimeric or absent.
(L) Flower with medial whorl 1 sepals converted into carpelloid organs; an ovule is visible (o).
(M) Medial whorl 1 sepals are staminoid (arrows), whereas lateral whorl 1 sepals (s) are normal. Whorl 2 petals are absent.

of class B and C genes reflects the homeotic transformations of floral organs as observed in the flowers of this double mutant.

LUG and *SEU* Expression Analysis

The floral phenotypes of the *agl24 svp* double mutant described above are strikingly similar to those observed in the *lug* and *seu* single and double mutants (Liu and Meyerowitz, 1995; Franks et al., 2002). In these mutants, the observed homeotic transformation of floral organ identity is due to precocious and ectopic class B and C gene expression. To investigate whether the expression of *LUG* and/or *SEU* was affected in the *agl24 svp* double mutant, RT-PCR analysis was performed on inflorescence RNA extracted from wild-type, *svp*, and *agl24* single and double mutants grown at 30°C. This analysis revealed that the expression levels in wild-type and mutant plants are comparable, which indicates that the observed phenotype in the *agl24 svp* double mutant is not due to the silencing of *LUG* and/or *SEU* (Figure 5).

Protein Interaction Analysis

Sridhar et al. (2004) have recently shown that the molecular basis for the similar mutant phenotype of *lug* and *seu* single mutants is based on the physical interaction between the LUG and SEU proteins in forming a corepressor complex. In this complex, LUG functions as the repressor of *AG* that acts via the second

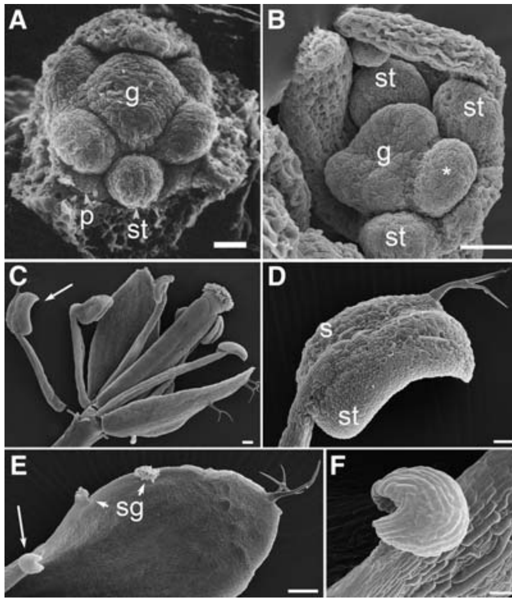

Figure 3. Scanning Electron Microscopy Analysis of Mutant Flowers.

(A) and **(B)** Scanning electron microscopy pictures of wild-type and *agl24 svp* double mutant flowers at 30°C.

(A) Lateral view of a wild-type bud at stage 6 (sepals that enclose the bud have been removed). The stamen primordia are clearly visible (st), whereas the petal primordia (p) are small. The gynoecium (g) is going to arise from the central dome. (*Copyrighted by the American Society of Plant Biologists and reprinted with permission from Bowman et al., 1989.*)

(B) *agl24 svp* double mutant bud at stage 6, in which only two sepal primordia and three normal stamen primordia (st) are visible. One stamen primordia (asterisk) is fused with the gynoecium primordia (g).

(C) to **(F)** Scanning electron microscopy pictures of *ap1agl24 svp* triple mutant flowers at 22°C.

(C) A triple mutant flower with medial whorl 1 staminoid sepals (arrow). Lateral whorl 1 sepals (s) are normal, and whorl 2 petals are absent.

(D) Close-up of whorl 1 organ mosaic for sepal (s) and stamen (st) tissues. Note the stamen filament at the base.

(E) A whorl 1 triple mutant organ mosaic for carpel and sepal tissues, with ovules developing from the margin of the sepal. Note the stigmatic tissue (sg).

(F) Close-up of an ovule developing from the margin.

Bars = 10 μm (**[A]** and **[B]**), 20 μm **(F)**, 50 μm **(D)**, and 100 μm (**[C]** and **[E]**).

regulatory intron (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000), whereas SEU does not seem to have any inherent function in repressing transcription. SEU is thought to function as an adapter protein bridging the interaction between the repressor LUG and specific DNA binding transcription factors.

Since there are two MADS box transcription factor binding sites (CArG boxes) in the regulatory intron of *AG* (Deyholos and Sieburth, 2000; Hong et al., 2003), we investigated whether SEU could directly interact with AGL24 and SVP. These interactions would explain how the LUG-SEU repressor complex is recruited to the DNA and by that the observed phenotypes in the *agl24 svp* double mutant.

To investigate this, yeast two-hybrid assays were performed using SVP, AGL24, SEU, and LUG since a direct interaction between LUG and the MADS box proteins could not be excluded. The coding part of the cDNAs encoding these proteins were fused to the activation domain (AD) and binding domain (BD) and tested for interaction (Table 2). SEU could not be tested in the BD vector since this protein has strong autoactivation activity. In this assay, we observed that LUG forms a strong interaction with SEU (growth on adenine selection and on His selection media with 5 mM 3AT), but neither SEU nor LUG showed an interaction with AGL24 and SVP.

Recently, Sridhar et al. (2004) indicated as unpublished data that SEU interacts with AP1 and SEPALLATA3 (SEP3). Interestingly, both SVP and AGL24 have been shown to interact with AP1 and SEP3 (Pelaz et al., 2001; de Folter et al., 2005), suggesting that SEU could interact with a complex composed of SVP or AGL24 and SEP3-AP1. Furthermore, Bowman et al. (1993) showed that strong *ap1* alleles develop carpels instead of sepals when these mutants were grown at high temperatures (25 to 30°C). This phenotype is similar to our *agl24 svp* double mutant; therefore, we used two-hybrid assays to confirm that AP1 and

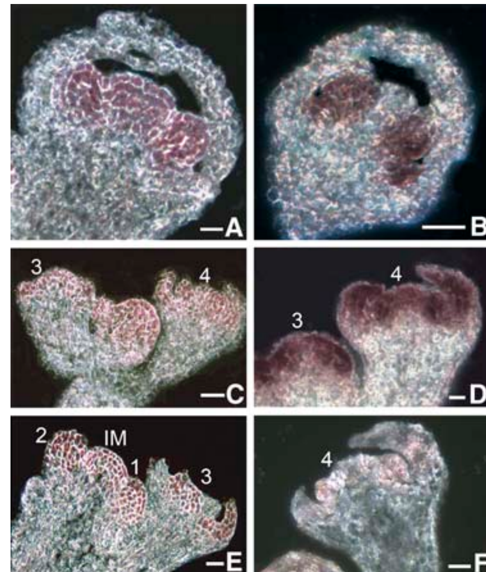

Figure 4. Class B and C Gene Expression in Wild-Type and *agl24 svp* Double Mutant Flowers.

In situ hybridization of digoxygenin-labeled antisense *AG* (**[A]**, **[C]**, and **[E]**) or *AP3* (**[B]**, **[D]**, and **[F]**) probes to 8-μm longitudinal sections of young wild-type and *agl24 svp* double mutant inflorescences. *AG* mRNAs are detected in earlier stages than in wild-type flowers, and *AG* and *AP3* expression is observed in all four whorls, indicating a deregulation of class B and C gene expression. Bars = 20 μm.

(A) Wild-type stage 6 flower. *AG* mRNA is only detected in the carpel and stamen primordia.

(B) Wild-type stage 6 flower. *AP3* mRNA is only detected in petal and stamen primordia.

(C) and **(E)** Inflorescences of the *agl24 svp* double mutant with flowers at different stages of development (stages 1 to 4) hybridized with the *AG* probe. IM, inflorescence meristem.

(D) and **(F)** Inflorescences of the *agl24 svp* double mutant with flowers at different stages of development hybridized with the *AP3* probe.

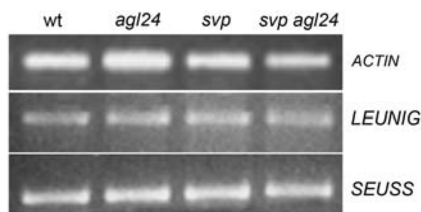

Figure 5. Expression of *LUG* and *SEU* Is Not Changed in the *agl24 svp* Double Mutant.

RT-PCR analysis of *LUG* and *SEU* using RNA extracted from inflorescences of wild-type, *agl24*, *svp*, and *agl24 svp* mutants. ACTIN expression was used as a control. The plants were grown at 30°C.

SEP3 interact with SEU. However, all these three proteins show high autoactivation activity when used as bait in the BD vector, making it impossible to test these interactions. To overcome this problem, we tested a deletion construct encoding 1 to 196 of AP1 (AP1Δ1), eliminating the transactivating terminus (Pelaz et al., 2001). This experiment showed that with the AP1Δ1 protein, no interaction with SEU was observed (Table 2), although this might be due to the loss of a part of the C terminus. An explanation for these results could be that either full-length AP1 can interact with SEU or a MADS box dimer composed of, for instance, AP1-AGL24, or AP1-SVP forms the surface for SEU interaction. To test whether at least the dimer is able to interact (which does not exclude the possibility that AP1 is establishing the interaction by itself), we performed yeast three-hybrid assays by fusing the full-length AP1 protein with the nuclear localization signal of the TFT vector (Egea-Cortines et al., 1999). Clear growth of single colonies was observed when the three proteins (SVP/AGL24, AP1, and SEU) were expressed, whereas all controls were clearly negative, showing that the AP1-AGL24 and AP1-SVP dimers can bind to SEU. However, when low concentrations of 3AT were added, no growth was observed, indicating that the interaction is weak (Table 2). To verify this weak interaction, we also performed semiquantitative lacZ assays using yeast strain SFY526, which has a different genetic background (see Supplemental Table 1 online). These assays confirmed the interaction between SEU and the AGL24-AP1 and SVP-AP1 dimers.

To have the ultimate evidence that the LUG-SEU corepressor can be recruited by a MADS box dimer, we developed a yeast four-hybrid assay in which we fused SVP to the BD domain, LUG to the AD domain, and both AP1 (in the TFT vector) and SEU (in the pRED vector) to a nuclear localization signal. Only when the four proteins were simultaneously expressed was growth on selective media observed, whereas all controls were clearly negative (Figure 6, Table 2), indicating that the four proteins indeed form a complex. These experiments support the idea that SEU mediates the interaction between the repressor protein LUG and the DNA binding MADS box dimers AP1-SVP and AP1-AGL24.

Analysis of the *ap1 agl24 svp* Triple Mutant

To reveal genetic evidence that AP1, together with AGL24 and SVP, is involved in the recruitment of the LUG-SEU corepressor complex, we combined the *svp agl24* mutant with the weak *ap1-12* allele (Figure 2J). Under standard growing conditions, the *ap1-12* mutant forms additional flowers that arise in the axils of the first-whorl sepals (Table 1). Furthermore, the number of petals is reduced in this mutant. To obtain information about the mutation that causes this phenotype, we sequenced the *AP1* cDNA, which showed that the *ap1-12* allele contains a nonsense mutation due to a single base pair change 547 bp downstream of the ATG.

In the *agl24 svp* double mutant, we only observed severe phenotypes similar to the *lug* mutant when plants were grown at 30°C (Figures 2E to 2I). Interestingly, in the *ap1 agl24 svp* triple mutant, severe *lug*-type phenotypes were observed under normal growing conditions (22°C) (Figures 2K to 2M). Whereas none of

Table 2. Interactions among MADS Box, LUG, and SEU Proteins in Yeast

Two-Hybrid Assay	-LWH	-LWH + 3AT
pBD-AGL24 + pAD-AGL24	+	+ (3 mM)
pBD-AGL24 + pAD-SVP	−	−
pBD-AGL24 + pAD-LUG	−	−
pBD-AGL24 + pAD-SEU	−	−
pBD-SVP + pAD-AGL24	−	−
pBD-SVP + pAD-SVP	−	−
pBD-SVP + pAD-LUG	−	−
pBD-SVP + pAD-SEU	−	−
pBD-AP1Δ1 + pAD-AGL24	+	+ (3 mM)
pBD-AP1Δ1+ pAD-SVP	+	+ (1 mM)
pBD-AP1Δ1+ pAD-LUG	−	−
pBD-AP1Δ1+ pAD-SEU	−	−
pBD-LUG + pAD-AGL24	−	−
pBD-LUG + pAD-SVP	−	−
pBD-LUG + pAD-LUG	−	−
pBD-LUG + pAD-SEU	+	+ (5 mM)
Three-Hybrid Assay	-LWAH	-LWAH + 3AT
pBD-AGL24 + pTFT-AP1 + pAD-SEU	+	−
pBD-AGL24 + pTFT-empty + pAD-SEU	−	−
pBD-AGL24 + pTFT-AP1 + pAD-empty	−	−
pBD-SVP + pTFT-AP1 + pAD-SEU	+	−
pBD-SVP + pTFT-empty + pAD-SEU	−	−
pBD-SVP + pTFT-AP1 + pAD-empty	−	−
Four-Hybrid Assay	-LWAUH	-LWAUH + 3AT
pBD-SVP + pTFT-AP1 pRED-SEU + pAD-LUG	+	−
pBD-empty + pTFT-AP1 pRED-SEU + pAD-LUG	−	−
pBD-SVP + pTFT-empty pRED-SEU + pAD-LUG	−	−
pBD-SVP + pTFT-AP1 pRED-empty + pAD-LUG	−	−
pBD-SVP + pTFT-AP1 pRED-SEU + pAD-empty	−	−

Interactions among SVP, AGL24, AP1, SEU, and LUG detected in yeast. cDNAs cloned in pBD are fused in frame with the DNA BD of GAL4, cDNAs cloned in pAD are fused to the GAL4 AD, and the cDNAs cloned in pTFT and pRED are fused to a nuclear localization signal. The yeast strains were plated on different selective media (without His) and with different concentrations of 3-aminotriazole (3AT). The minus sign indicates no growth and the plus sign indicates growth on selective media.

the segregants (single or double mutant combinations) showed these kind of severe phenotypes, scanning electron microscopy analysis shows clearly that the whorl 1 organs are converted into carpelloid structures on which ovules and stigmatic tissue develops (Figures 3E and 3F) or into chimeric organs composed of sepal and stamen tissues, as previously observed in the *lug* mutant (Figures 3C and 3D). These phenotypes make clear that combining a mild *ap1* allele with the *agl24 svp* double mutant significantly enhances the phenotype. These data suggest that *AP1*, together with *SVP* and *AGL24*, indeed regulates *AG*.

DISCUSSION

The *agl24 svp* Double Mutant Phenocopies *lug* and *seu* Mutant Phenotypes

Functional redundancy between homologous MADS box genes seems to be a common feature (Pařenicová et al., 2003). The phylogenetic analysis of all *Arabidopsis* MADS box factors is a helpful tool to predict these redundancies. This analysis grouped the MADS box factors SVP and AGL24 closely together, indicating that the *AGL24* and *SVP* genes are the result of a gene duplication event and therefore might have redundant functions. Surprisingly, the single mutant phenotypes indicated the contrary, since *SVP* and *AGL24* have opposite functions in the control of flowering time. In this study, we combined the two mutants and carefully analyzed the *agl24 svp* double mutant plants (Table 1). Concerning flowering time, the *agl24 svp* double mutant flowers as early as the *svp* single mutant, indicating that

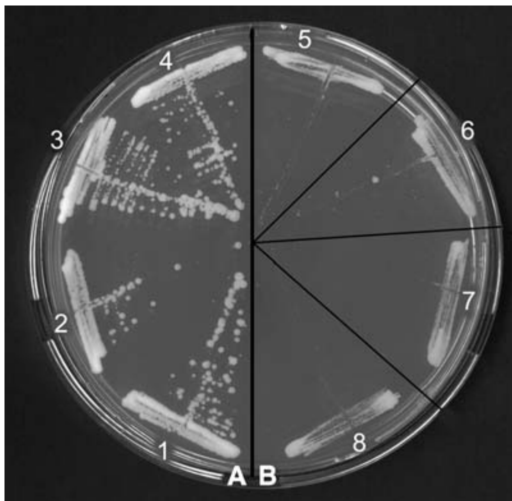

Figure 6. Interactions among the SVP, AP1, SEU, and LUG Proteins in Yeast.

Yeast four-hybrid assays using selective media (without His).

(A) Four independent yeast transformants cotransformed with pBD-SVP, pTFT-AP1, pRED-SEU, and pAD-LUG (1 to 4, respectively).

(B) Controls in which yeast was cotransformed with the same four constructs as in **(A)**, but in each control one vector is empty: (5) pAD-empty, (6) pRED-empty, (7) pTFT-empty and (8) pBD-empty.

SVP is epistatic to *AGL24*. Analysis of the flowers of this double mutant showed that *AGL24* and *SVP* have indeed a redundant function as predicted by phylogenetic analysis. The observed phenotype was mild under standard growing conditions (22°C) but was significantly enhanced when the plants were grown at 30°C, indicating a temperature-sensitive effect. The phenotypes observed at 30°C were very similar to those reported for the *lug* and *seu* mutants (Liu and Meyerowitz, 1995; Conner and Liu, 2000; Franks et al., 2002; Sridhar et al., 2004). Typical for these mutants are homeotic conversion of the sepals into staminoid and carpelloid structures, and petals are staminoid or absent. In situ hybridization analysis of *agl24 svp* double mutant flowers showed that the class C gene *AG* is expressed earlier and that both *AG* and the class B gene *AP3* are not restricted to specific floral whorls. This misexpression is also observed in the *lug* and *seu* mutants and explains the observed phenotypes. Another similarity between these mutants is that the misexpressed genes are often expressed in patches, which probably results in the observed mosaic floral organs with various identities.

The LUG and SEU proteins are considered to form a corepressor complex that prevents *AG* expression in the outer two whorls during flower development (Conner and Liu, 2000; Franks et al., 2002; Sridhar et al., 2004). Combining *lug* and *ag* mutants showed that the ectopic B activity was strongly reduced in this double mutant, indicating that ectopic *AG* activity in the *lug* single mutant induces misexpression of class B genes (Liu and Meyerowitz, 1995). Besides *AG*, *LUG* seems to regulate several other target genes since defects like narrow leaves, sepals, and petals, split stigma, and abnormal carpels and ovules observed in *lug* mutants are independent of *AG* (Conner and Liu, 2000). Interestingly, these phenotypic effects independent of *AG* ectopic expression are not observed in the *agl24 svp* double mutants. We rarely observed partially fused carpels, but this is likely due to the formation of staminoid tissue. This probably indicates that the *AGL24* and *SVP* genes are mainly involved in *AG* repression and not in other processes in which *SEU* and *LUG* are involved.

The repression of *AG* by *SEU* and *LUG* seems to act early in flower development, like has been observed for *AP2*, whereas the repressive function of the polycomb group gene *CURLY LEAF* (*CLF*) is later in development, which is also reflected by the milder phenotypes of *clf* mutants (Goodrich et al., 1997). *SVP* and *AGL24* are both expressed during early stages of flower development (*SVP* until stage 3; sepal primordia visible as shown in Hartmann et al., 2000). The severe phenotypic effect observed in the *agl24 svp* double mutant suggests a repressive role for these MADS box factors also during later stages of development (at least until around stage 5). An explanation for this might be that after stage 3, the expression of *SVP* is too low to be detected by in situ hybridization or that this MADS box protein is stable enough to control *AG* expression during subsequent stages of flower development. A more likely explanation might be that due to the absence of *AGL24* and *SVP* at very early stages of flower development, there is a precocious accumulation of AG and other floral identity factors that could deregulate negative and positive feedback loops that control flower development, causing effects on later stages of flower development (de Folter et al., 2005; Gómez-Mena et al., 2005).

MADS Box Dimer Interacts in Yeast with the SEU-LUG Corepressor

LUG encodes seven WD repeats, a LUFS motif, and two Q-rich regions (Conner and Liu, 2000). SEU interacts directly with the LUFS domain of LUG, although for the interaction with LUG, the entire SEU protein is needed (Sridhar et al., 2004). LUG is similar in motif structure to the yeast corepressor Tup1, and *SEU* encodes a plant-specific regulatory protein with sequence similarity to Ssn6. In yeast, Ssn6 functions as an adaptor protein bridging the interaction between Tup1 and specific DNA binding transcription factors (Smith and Johnson, 2000). Recent results of Pfluger and Zambryski (2004) suggest that SEU might have a similar function. They showed that SEU physically interacts with ETTIN, a transcription factor belonging to the auxin response factor family, probably bridging the interaction with other regulatory molecules to modulate transcription of auxin response genes. SEU seems to function in a similar way to bridge the interaction between LUG and DNA binding factors. Genetic studies have shown that intragenic regions of *AG* are essential for the regulation by *LUG* (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000). Candidate factors that could recruit the LUG-SEU corepressor complex to DNA are BELLRINGER (BLR) and MADS box proteins, the latter because of the presence of two CArG boxes in the *AG* regulatory intron. *BLR* has been shown to act as a repressor of *AG* and directly binds to the *AG* intron. Furthermore, *blr* shows synergistic genetic interactions with *lug* and *seu*, which makes it a perfect candidate (Bao et al., 2004). We assayed interactions between BLR and SEU or LUG in yeast, but we did not observe direct interactions between these proteins (data not shown).

The yeast two-hybrid assays using AGL24 and SVP also showed that these MADS box factors do not directly recruit SEU or LUG to the DNA. Since AP1 was suggested to interact with SEU (Sridhar et al., 2004) and because AP1 also interacts with SVP and AGL24 (de Folter et al., 2005), we also tested AP1 for interactions with SEU and LUG. These assays showed, using a truncated version of AP1 (AP1Δ1) due to autoactivation of this protein, that AP1Δ1 does not interact with SEU or LUG. Subsequently, we tested the MADS box dimers AP1-AGL24 and AP1-SVP for interactions with the SEU-LUG corepressor by yeast three- and four-hybrid assays. These experiments revealed that MADS box dimers (AP1-AGL24 and AP1-SVP) interact weakly in yeast with the corepressor, which supports the idea that SEU mediates the interaction between the repressor protein LUG and the DNA binding MADS box dimers AP1-SVP and AP1-AGL24. Future experiments will have to reveal whether these MADS box corepressor complexes indeed directly bind to the CArG boxes that are located in the *AG* regulatory intron.

To reveal genetic evidence that AP1, together with AGL24 and SVP, is involved in the recruitment of the LUG-SEU corepressor complex, we combined the *svp agl24* mutant with the weak *ap1-12* mutant (Table 1). Interestingly, in the *agl24 svp* double mutant, we only observed severe phenotypes similar to the *lug* mutant when plants were grown at 30°C, whereas in the *ap1 agl24 svp* triple mutant, severe *lug*-type phenotypes were observed under normal growing conditions. These data suggest that AP1 indeed recruits, together with SVP and AGL24, the SEU-LUG corepressor complex for the regulation of *AG*. This is further supported by the fact that the *ap1-1* mutant enhances floral homeotic transformation and *AG* misexpression in the *lug-1 ap1-1* double mutant (Liu and Meyerowitz, 1995).

Interestingly, mutant alleles of *AP1*, *BLR*, or a combination of *SVP* and *AGL24*, which all encode putative partners of a complex that recruits the SEU-LUG repressor to regulate *AG*, cause a temperature-sensitive phenotypic effect (Bowman et al., 1993; Bao et al., 2004). It seems that when one of the components of this complex is missing, the remaining factors can still recruit the repressor complex. However, at higher temperatures, the incomplete complex might get unstable, and *AG* repression is lost. In the *ap1 agl24 svp* triple mutant, the absence of three essential factors can probably not be compensated; therefore, *AG* repression is also lost at normal growing temperatures.

Molecular Mechanism underlying the ABC Model

The ABC model of flower development explains how three classes of genes control sepal, petal, stamen, and carpel identity and predicts that class A genes control sepal and petal identity (Coen and Meyerowitz, 1991). Furthermore, the model indicates that class A and C genes are mutually antagonistic, which means that class A genes prevent C expression in the outer two whorls and vice versa (Drews et al., 1991). In *Arabidopsis*, two class A genes have been identified, which are *AP1* and *AP2*. Typically, the *ap1* mutant forms bracts in stead of sepals, and petals mostly do not develop (Irish and Sussex, 1990). Furthermore, in the axil of the first-whorl organs, a new *ap1* flower develops. The *ap2* mutant also rarely develops petals, but in this mutant, sepals are transformed into carpelloid structures due to ectopic *AG* expression. These phenotypic observations resulted in the generally accepted idea that *AP2* is the key player in preventing *AG* expression in whorls 1 and 2 (Kunst et al., 1989). Liu and Meyerowitz (1995) suggested that *AP1* is likely a redundant repressor of *AG* since *ap1-1* enhanced floral homeotic transformations in *lug-1 ap1-1* and *ap1-1 ap2-1* double mutants. Our results confirm this role of *AP1* in the control of *AG* expression and show that *AP1* is redundant for this function with *SVP* and *AGL24*.

Interestingly, in *ap2* mutants, *AG* deregulation seems to start from around stage 3 of flower development (Drews et al., 1991). In the *agl24 svp* double mutant, *AG* was deregulated at earlier stages (1 to 3). This suggests that *AP1*, *AGL24*, and *SVP* are involved in *AG* repression in the first stages of flower development, whereas *AP2* seems to act later, restricting *AG* expression to the inner two whorls.

The data presented here show that MADS box factors play different roles in the developmental pathway that finally leads to plant reproduction. For instance, in the vegetative phase, high levels of *SVP* expression repress the transition to flowering (Hartmann et al., 2000); however, when its expression reduces and *AGL24* expression increases, the floral transition is promoted and then *AGL24* promotes inflorescence identity (Yu et al., 2002; Michaels et al., 2003). To establish floral meristem identity, *AGL24* is repressed by *AP1* (Yu et al., 2004). Subsequently, all three factors have a function in the repression of *AG* in the floral meristem. The diversity in function of these MADS box factors

is probably obtained by making different protein–protein interactions. This all illustrates the complexity of the regulation of developmental processes and how transcription factors are recycled for different functions.

METHODS

Plant Material and Growth Conditions

The plants were grown at 22 or 30°C under SD (8 h light/16 h dark) or long-day conditions (16 h light/8 h dark). The *agl24-2* and *svp-41 Arabidopsis thaliana* mutants (ecotype Columbia) have been kindly given by R.M. Amasino and P. Huijser, respectively. The *agl24-2* allele is an En transposon line, and genotyping of the alleles was performed as described previously (Michaels et al., 2003). In the *svp-41* mutant, a 2-bp deletion causes a frame shift (Hartmann et al., 2000). Genotyping of *SVP* alleles was performed by PCR using the gene-specific oligonucleotides 198 and 199 for the wild type (5′-GACCCACTAGTTATCAGCTCAG-3′ and 5′-AAGTTATGGCTCTCTAGGAC-3′) and oligonucleotide 200 designed on the mutation (5′-AAGTTATGGCTCTCTAGGTT-3′). Seeds from the *ap1-12* mutant in Columbia were obtained from the Nottingham Arabidopsis Stock Centre.

Expression Analysis

For the in situ hybridization, *Arabidopsis* flowers were fixed and embedded in paraffin as described previously (Lopez-Dee et al., 1999). Digoxigenin-labeled gene-specific antisense RNA probes were generated by in vitro transcription following the instructions of the in vitro transcription kit (Roche). Hybridization and immunological detection were performed as described previously (Lopez-Dee et al., 1999).

Total RNA was extracted from *Arabidopsis* tissues using the SV total RNA isolation system (Promega). RT-PCR reactions were performed as described previously (Lago et al., 2004) using primers AtP536 and AtP537 for *SEU* (5′-GAAGACTTTTGATACCGCAGG-3′ and 5′-TGCAGATGAAGGGCCTGTTCTC-3′) and AtP531 and AtP532 for *LUG* (5′-CTTAAGTTAAAGATGGCTCTG-3′ and 5′-TCAACATTGTCGTCAAGTGATCC-3′).

Yeast Two-, Three-, and Four-Hybrid Assays

The two-, three-, and four-hybrid assays were performed in the yeast strains PJ69-4A and SFY526 as described previously (Davies et al., 1996; James et al., 1996). pBD, pAD, pTFT1, and pRED vector constructs were selected on YSD media lacking Leu, Trp, adenine, and uracil, respectively. Three-hybrid interactions were assayed on selective YSD media lacking Leu, Trp, adenine, and His supplemented with different concentrations of 3AT (1, 3, or 5 mM). Four-hybrid interactions were assayed on selective YSD media lacking Leu, Trp, adenine, uracil, and His supplemented with different concentrations of 3AT (1, 3, or 5 mM). Genes used for the yeast two-, three-, and four-hybrid assays were cloned in the Gateway vector GAL4 system (pDEST32 for BD and pDEST22 for AD) passing through pDONOR201 (Life Technologies) as described by de Folter et al. (2005). The coding sequences of *LUG* and *SEU* were amplified using primers AtP524 and AtP525 for *LUG* (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTCAGACCAACTGG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATAGTTTTCACTTCCACAG-3′) and AtP526 and AtP527 for *SEU* (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTACCATCAGAGCCGCCTAATCC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCATTTCACGCGTTCC-3′).

Scanning Electron Microscopy

Samples were prepared and analyzed as described previously (Favaro et al., 2003).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At2g22540 (*SVP*), At4g24540 (*AGL24*), At1g69120 (*AP1*), At1g43850 (*SEU*), and At4g32551 (*LUG*).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. Semiquantitative Yeast Three-Hybrid Assays.

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