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The authors mistakenly cited Lenhard et al., 2001 instead of Lohmann et al., 2001 in the above paper. The citation appears in the last paragraph on p. 3165. The correct reference is shown below.


The authors apologise to readers for this mistake.
APETALA1 and SEPALLATA3 interact with SEUSS to mediate transcription repression during flower development

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The transcriptional repression of key regulatory genes is crucial for plant and animal development. Previously, we identified and isolated two Arabidopsis transcription co-repressors LEUNIG (LUG) and SEUSS (SEU) that function together in a putative co-repressor complex to prevent ectopic AGAMOUS (AG) transcription in flowers. Because neither LUG nor SEU possesses a recognizable DNA-binding motif, how they are tethered to specific target promoters remains unknown. Using the yeast two-hybrid assay and a co-immunoprecipitation assay, we showed that APETALA1 (AP1) and SEPALLATA3 (SEP3), both MADS box DNA-binding proteins, interacted with SEU. The AP1-SEU protein-protein interaction was supported by synergistic genetic interactions between AP1 and SEU mutations. The role of SEU proteins in bridging the interaction between AP1/SEP3 and LUG to repress target gene transcription was further demonstrated in yeast and plant cells, providing important mechanistic insights into co-repressor function in plants. Furthermore, a direct in vivo interaction of SEU proteins with the AG cis-regulatory element was shown by chromatin immunoprecipitation. Accordingly, a reporter gene driven by the AG cis-element was able to respond to AP1- and SEP3-mediated transcriptional repression in a transient plant cell system when supplied with SEU and LUG. These results suggest that AP1 and SEP3 may serve as the DNA-binding partners of SEU/LUG. Our demonstration of the direct physical interaction between SEU and the C-terminal domain of SEP3 and AP1 suggests that AP1 and SEP3 MADS box proteins may interact with positive, as well as negative, regulatory proteins via their C-terminal domains, to either stimulate or repress their regulatory targets.

KEY WORDS: AGAMOUS (AG), Co-repressors, MADS box proteins, Transcription repression, Flower

INTRODUCTION

The genetic control of floral organ identity is one of the most important examples of how regulatory genes determine plant structure. Specific combinations of the A, B, C and E classes of genes direct the development of sepal, petals, stamens and carpels in four concentric floral whorls, respectively (Coen and Meyerowitz, 1991; Jack, 2004; Theissen and Saedler, 2001). Almost all ABCE genes encode the MIK-type MADS box transcription factors that can interact with each other to form multimeric complexes for gene activation (Honma and Goto, 2001; Theissen and Saedler, 2001). It was proposed that four different multimeric protein complexes consisting of A/E, A/B/E, B/C/E and C/E protein combinations control the four organ type-specific development (Theissen and Saedler, 2001). An important aspect of this model is that the A, B and C class genes are only transcribed in specific floral whorls; their expression correlates with the domain of their function. The domain or whorl-specific expression of the A, B and C genes therefore underlies the formation of whorl-specific MADS box complexes.

Previously, we identified and characterized two transcription corepressors, LEUNIG (LUG) and SEUSS (SEU), that play crucial roles in preventing ectopic expression of the class C gene AGAMOUS (AG) (Franks et al., 2002; Liu and Meyerowitz, 1995). In wild type, AG is expressed in the inner two whorls of a flower to specify stamen and carpel development (Bowman and Meyerowitz, 1991; Drews et al., 1991; Yanofsky et al., 1990). The ectopic expression of AG in lug or seu mutants in all four floral whorls causes partial homeotic transformations of whorl 1 sepals into carpelloid sepals, and whorl 2 petals into staminoid organs or organ loss. lug and seu exhibited a synergistic genetic interaction causing a more complete homeotic transformation from sepals to carpels, and a more severe reduction of floral organs in lug seu double mutants (Franks et al., 2002; Liu and Meyerowitz, 1995), suggesting that LUG and SEU are partially redundant in controlling AG expression.

LUG encodes a nuclear protein with an overall domain structure similar to a class of functionally related transcriptional co-repressors, including Tup1 of yeast and Groucho of Drosophila (Conner and Liu, 2000; Hartley et al., 1988; Williams and Trumbly, 1990). Additionally, LUG possesses a conserved N-terminal 88-amino acid domain named the LUFs domain. The N-terminal half of the LUFs domain corresponds to the Lis1-homologous (LisH) domain, which was originally identified in a series of proteins associated with human disease (Emes and Ponting, 2001), and was subsequently shown to promote dimerization, tetramerization and interaction with other proteins (Cerna and Wilson, 2005). SEU encodes a glutamine (Q)-rich protein with a conserved domain that is similar to the dimerization domain of the LIM-domain-binding (Ldb) family of transcriptional co-regulators, such as the Ldb1 in mouse and Chip in Drosophila (Franks et al., 2002). SEU was shown to directly interact with the LUFs domain of LUG (Sridhar et al., 2004) and may form a co-repressor complex with LUG in Arabidopsis (Franks et al., 2002; Sridhar et al., 2004). This complex is likely to be evolutionarily conserved, as a direct interaction between STYLOSA (STY), the ortholog of LUG in Antirrhinum, and AmSEUSS was reported (Navarro et al., 2004).

Strong repressor activity of LUG was demonstrated by tethering LUG to heterologous promoters of reporter genes via the GAL4 DNA-binding domain (BD) in yeast and in plant cells (Sridhar et al., 2004). The repressor activity of LUG was shown to depend on histone deacetylases (Sridhar et al., 2004). By contrast, SEU exhibited no repressor activity when it was similarly tethered to the
heterologous promoters of reporter genes via the GAL4-BD. Because neither LUG nor SEU possesses a recognizable DNA-binding motif, how they are tethered to specific target promoters in vivo remains unknown. Furthermore, because LUG and SEU are broadly expressed in both flowers and vegetative tissues (Conner and Liu, 2000; Franks et al., 2002), how LUG and SEU confer their outer whorl-specific repressor activity on AG is unknown. One attractive model that addresses both of these questions is that the putative LUG/SEU complex interacts with DNA-binding partners that are specifically expressed in the outer two whorls of a flower. A second model is that LUG/SEU may regulate AG indirectly by repressing the expression of positive regulators of AG. A third model proposes that SEU/LUG represses AG in all four floral whorls, and some factors in the inner two whorls are able to antagonize the repressor effect of LUG/SEU.

APETALA1 (AP1) and SEPARATE3 (SEP3), both MADS box proteins, belong to the A and E class floral organ identity genes, respectively, and have been shown to activate the expression of B and C class genes (Castillejo et al., 2005; Gomez-Mena et al., 2005; Weigel and Meyerowitz, 1993). In this study, we demonstrate a direct SEU-AP1 and SEU-SEP3 protein-protein interaction, as well as synergistic genetic interactions between seu and ap1 mutations, indicating that AP1 and SEP3 may act as the DNA-binding partners of LUG/SEU. In vivo association of SEU to the AG cis-regulatory elements shown by chromatin immunoprecipitation eliminates the second model of an indirect role of SEU/LUG in AG regulation. A revised third model is proposed illustrating how the outer whorl-specific repression of AG is achieved.

MATERIALS AND METHODS

Yeast two-hybrid assay

The yeast strains PJ69-4A (James et al., 1996) and SEU-BD have been described previously (Sridhar et al., 2004). AP1-AD and SEP3-AD in pACT2 (Honma and Goto, 2001), PI-AD (pDI293) in pGAL4-C and AP3-AD (pDI294) in pGDBU-C (Yang et al., 2003) were gifts from Drs K. Goto and T. Jack, respectively.

To construct AP3-AD, AP3 was excised from pDI294 as a BamHI/PstI fragment and inserted into pGAL4-C at the same sites. AP1-MIK and SEP3-MIK were excised from the AP1-AD and SEP3-AD in pACT2 (Honma and Goto, 2001) as an Ncol/Xhol fragment and cloned into pET30a (Novagen) at the same sites. Subsequently, the respective MIK fragment was excised as an Ncol/Xhol fragment from above pET30a-MIK and inserted into pGADT7 (Clontech). AP1-C and SEP3-C were excised from the AP1-AD and SEP3-AD in pACT2 (Honma and Goto, 2001) as a SacI/Xhol fragment, and cloned into pET30a and pET30c at the same sites, respectively. Subsequently, AP1-C and SEP3-C were excised as an Ncol/Xhol fragment and cloned into pGADT7 (Clontech).

The yeast two-hybrid assay was performed as previously described (Sridhar et al., 2004). β-galactosidase activity was measured in triplicate using the Galacto Light Plus Kit (Applied Biosystems) and normalized with the OD of the culture.

Yeast repression assay

Full-length SEU cDNA was excised from HHFL#7 (Sridhar et al., 2004) with HindIII/Xhol and inserted into p26GALL (Mumberg et al., 1994). As a result, SEU was driven by the GALL promoter and its expression is induced by galactose. LUG and LUG_{dul} were expressed from pGAD424 (Clontech) but with the GAL4-AD domain removed. Specifically, Kpor and Smol were used to digest LUG-AD and LUG_{dul} to LUG_{dul}-AD in pGAD424 (Sridhar et al., 2004), and relocated to delete the GAL4-AD. The pGAD424 control vector was similarly treated to remove the GAL4-AD from pGAD424 (Clontech). AP1-AD in pAS2-I was a gift from Dr K. Goto (Honma and Goto, 2001). SEP3-AD was constructed by digesting SEP3 in pACT2 (Honma and Goto, 2001) with Ncol/XhoI. The Ncol/XhoI fragment was cloned into pGBKT7 (Clontech) at the Ncol/SalI sites.

For yeast strain PJ69-4A (James et al., 1996), yeast transformation and β-galactosidase assay were similarly performed as the yeast two-hybrid assay. AP1-AD or SEP3-AD was selected by –Trp, and LUG or LUG_{dul} was selected by –Leu. SEU was selected by Ura3, and its expression was induced by the addition of galactose instead of glucose in the media. Data shown in Fig. 3A are averages of triplicates, and the experiment was repeated twice.

In vitro pulldown assay

His-tagged AP1 and AP3 were gifts from Dr X. Chen (U.C. Riverside). Full-length AP1 and AP3 cDNAs were cloned into the EcoRI/BamHI sites in pRESET (Invitrogen). API-C and SEP3-C were excised from AP1-AD and SEP3-AD in pACT2 (Honma and Goto, 2001) as a SacI/Xhol fragment and cloned into pET30a and pET30c (Novagen), respectively. Full-length SEP3 was excised from SEP3-AD in pACT2 as an Ncol/Xhol fragment and cloned into the same sites of pET30a (Novagen).

Plasmid templates were used for synthesizing 35S-radiolabeled AP1, SEU and AP3 proteins with the TnT Quick Coupled Transcription/Translation System (Promega). The TnT reaction mix (5 μl) was loaded directly onto a NuPAGE gel (Invitrogen) as the input control. For two-protein pulldown, GST-SEU protein was purified from bacteria as previously described (Sridhar et al., 2004). GST-SEU protein (5 μg) bound on the GST.Bind resin (Novagen) was incubated with 15 μl 35S reaction mix from TnT for 2 hours at room temperature. The GST-SEU bound resin was washed five times with cold 1×PBS, resuspended in protein gel loading buffer, heated and loaded directly to the NuPAGE gel, and run for 3 hours at 70 V.

For three-protein pulldown, MBP and MBP-LUFS proteins were purified from bacteria as previously described (Sridhar et al., 2004). MBP-LUFS proteins were bound to amyllose beads. After an extensive wash with cold 1×PBS, 10 μl MBP-LUFS/amyllose beads were estimated for protein concentration. MBP-LUFS/amyllose beads (5 μg) were then incubated at 4°C for two hours with 5 μg SEU-GST eluted from the GST.Bind resin, as well as 15 μl 35S-labeled AP1, SEU or AP3 from TnT. The MBP-LUFS/amyllose resin was washed five times with cold 1×PBS, resuspended in protein gel loading buffer, heated and then loaded onto the NuPAGE gel.

Repression assays in plants

To construct the pAG3:::LUC reporter, primer pair AGdF (5’-TGGTCTGCTTCTACGATCC-3’) and AGdR (5’-TATAAATTCTGGCAACCGATCC-3’) was used to amplify the –900 bp AG3’ enhancer using Columbia genomic DNA as a template. The PCR product was TA-cloned into pCRII-TOPO (clone #27). The –900 bp AG fragment was excised from clone #27 with KpnI/Xhol and inserted into –58LUC, a gift from Dr Krizek. –58LUC was constructed by inserting the –58/+6 fragment of the 35S promoter into the BGIII site of pGL3 (Promega), destroying the BGIII site (Krizek and Sulli, 2006).

Full-length AP1 and SEU were excised as an Ncol/Xhol fragment from their respective pACT2 plasmids (Honma and Goto, 2001) and inserted into the Ncol/Xhol sites of pSPUTK (Stratagen). AP1 and SEP3 were subsequently excised from pSPUTK as a HindIII/Xhol fragment, and cloned into pART7 (Gleave, 1992) at the same sites to yield 35S::AP1 and 35S::SEP3. 35S::LUG was described previously (Sridhar et al., 2004). 35S::SEU was constructed by excising SEU from HFFL#7 (Sridhar et al., 2004) as a SalI/Xhol fragment and inserted in pART7 at the same sites.

One microgram of pAG3:::LUC, 100 ng 35S::Renilla LUC, 1 μg 35S::AP1 (or 35S::SEP3), 1 μg 35S::SEU and 2 μg of 35S::LUG were mixed and introduced into onion epidermal cells by particle bombardment following the protocol of Padmanabhan et al. (Padmanabhan et al., 2005). Total DNA was maintained constant at 5 μg per transformation by using the pART7 vector to make up the difference between transformations. Diced onion was bombarded and followed by incubation at room temperature for 16-20 hours in a petri dish containing wet 3MM paper. Epidermal peels were removed from the onion, ground, lysed with Passive Lysis Buffer and assayed with the Dual-Luciferase Reporter Assay System (Promega). The data shown in Fig. 3C are averages of triplicate assays. The experiment was repeated twice.
Genetic analyses

seu-1 ap1-1 and seu-1 ap1-3 double mutants were constructed by crossing ap1-1 or ap1-3 with seu-1. Seeds of ap1-like F2 plants were individually collected. These ap1-like plants can be divided into three types. Type I plants were ap1 single mutants, which produced 100% ap1 in F3. Type II plants exhibited a more severe phenotype than ap1 single mutants and segregated three types of progeny in F3, suggesting that type II were homozygous for ap1 but heterozygous for seu-1. Type III plants exhibited the most severe phenotype and produced F3 progeny that all resembled their parent, indicating that type III is homozygous for ap1-1 and seu-1. The segregation patterns of these mutants were verified in F4.

Chromatin immunoprecipitation

The anti-SEU chicken antibody (AA126-139A), a gift of Dr Franks, was raised by Gallas ImmunoTech against a peptide (CNQLLAEQ-QRKKMEKLH) located at the N-terminal domain of SEU. To purify the anti-SEU antibody, 20 μg purified SEU-GST from bacteria was spotted onto nitrocellulose membrane and then blocked by 10% milk in 0.01% Tween-TBS (TTBS) for 1 h at room temperature. The membrane was washed five times with TTBS and incubated with 1 ml crude anti-SEU serum overnight at 4°C. After an extensive wash with TTBS, the bound antibody was eluted with 500 μl 0.2 M Glycine (pH 2.8) and immediately neutralized with 50 μl 1 M Tris-HCl (pH 8.0). The purified antiserum was concentrated to 20 μl using the Micon centrifugal filter device (Millipore).

Chromatin immunoprecipitation was performed essentially as described by Kwon et al. (Kwon et al., 2005). Wild-type (Ler, 1.8 g) and seu-3 (1.8 g) inflorescences were fixed with 1% formaldehyde for 2 hours. One-twentieth of the eluted DNA was used for PCR (94°C for 1 minute; then 35 cycles of 94°C for 15 seconds, 55°C for 40 seconds and 72°C for 40 seconds). The AG-3 primer pair (5'-CTATGTACAAGTACATACAGGAAACTC-3' and 5'-GATAGGTCAATCGACCTTGGCACAG-3') amplifies the 3' AG second intron. The AG-5 primer pair (5'-GCCTGTGTCGTCTTATGAGTACTCTAAC-3' and 5'-CTCCACATTAGAAAAACCTGATGGG-3') amplifies the 5' AG second intron. The control E1F4P primer was based on that described by Kwon et al. (Kwon et al., 2005).

RESULTS

SEU interacts with AP1 and SEP3 in yeast and in vitro

To test our first model on how the putative LUG/SEU complex may interact with a whorl-specific floral gene product with DNA-binding activities, we performed a yeast two-hybrid assay against several DNA-binding transcription factors in flower development, including AP1, APETALA2 (AP2), LEAFY (LFY) and HELLEBOREDIN (BRL) (Bao et al., 2004; Jofuku et al., 1994; Mandel et al., 1992; Weigel et al., 1992). Although LUG failed to interact with any of the proteins tested (data not shown), SEU interacted strongly with AP1 (Fig. 1A). To test the specificity of the interaction between SEU and AP1, other MADS box genes belonging to the B and E classes were subsequently tested. A strong interaction between SEU and SEP3 was found (Fig. 1A). However, an interaction between SEU and AP3 or PI was not detected (Fig. 1A). The interaction between SEU and AP1 or SEP3 protein was confirmed by the in vitro pull-down assay. 35S-labeled AP1, SEP3 or AP3 was bound to SEU-GST resin. After washing, AP1 and SEP3, but not AP3, were retained by the SEU-GST resin (Fig. 1C).

To determine the domain of AP1 or SEP3 that interacts with SEU, AP1 and SEP3 were each divided into the N-terminal MIK (MADS-box, Intervening region, and K-box) domain and the C (carboxyl) domain. In yeast, SEU failed to interact with either AP1-MIK or SEP-MIK but interacted with AP1-C and SEP3-C (Fig. 1B). The interaction of SEU with the C terminus of AP1 or SEP3 is weaker than with the full-length AP1 or SEP3 (compare Fig. 1A with 1B). This weaker interaction is confirmed by the in vitro pull-down assay with SEU-GST serving as a bridging protein. The ability of MBP-LUFS/amylose beads to retain 35S-labeled AP1, SEP3 or AP3 was tested in the presence (+) or absence (–) of SEU-GST. MBP was used as a negative control.
vitrin pull-down assay (Fig. 1C). There are two possible explanations for the weak interaction between SEU and AP1-C or SEP3-C. First, AP1-C or SEP3-C protein truncations may be less stable than full-length proteins. Second, AP1 or SEP3 may require homo- or heterodimerization in order to strongly interact with SEU. Dimerization is likely to be absent for these C-terminal truncations.

The C-terminal domain is the most divergent domain of MADS box proteins with no obvious sequence similarity among AP1, SEP3, AP3 and PI except that AP1-C and SEP3-C are both rich in glutamine (Q). It remains to be seen if the glutamines in AP1-C and SEP3-C are important for the interaction with SEU, which is also Q-rich. The absence of interaction between SEU and the B class proteins AP3 or PI suggests that SEU may specifically interact with a subset of MADS box proteins, including AP1 and SEP3. However, our assay could not exclude the possibility that the AP3/PI heterodimers maybe able to interact with SEU.

**SEU bridges an interaction between LUG and AP1/SEP3**

If SEU but not LUG interacts with AP1 and SEP3, could SEU bridge the interaction between LUG and AP1 or SEP3? This was tested by in vitro pull-down assays using the LUFS domain of LUG tagged by Maltose Binding Protein (MBP). The LUFS domain of LUG was previously shown to be necessary and sufficient for interacting with SEU (Sridhar et al., 2004). The interaction between LUFS-MBP and 35S-labeled AP1, SEP3 or AP3 was tested in the presence or absence of purified SEU (Fig. 1D). In the absence of SEU, LUFS-MBP/amylose resin failed to retain any of the 35S-labeled AP1, SEP3 and AP3 proteins. By contrast, when SEU was added, 35S-labeled AP1 and SEP3, but not 35S-labeled AP3, were retained by the LUFS-MBP/amylose resin. The interaction is specific to LUFS, as MBP alone failed to interact with AP1 or SEP3, even in the presence of SEU (Fig. 1D).

**Genetic interactions between ap1 and seu**

If AP1 is a DNA-binding partner of LUG/SEU in AG repression, and partial functional redundancies exist among AP1, LUG and SEU, ap1 mutations may exhibit a synergistic genetic interaction with lug and seu. A synergistic genetic interaction between lug-1 and ap1-1 was previously reported (Liu and Meyerowitz, 1995). A more dramatic transformation from sepals to carpels and a more severe reduction of whorl 2-3 organs were observed in lug-1 ap1-1 double mutants. The enhanced phenotype correlated with an increased ectopic AG expression in the lug-1 ap1-1 double mutants (Liu and Meyerowitz, 1995).

We constructed seu-1 ap1 double mutants using weak ap1-3 (Fig. 2C) and strong ap1-1 (Fig. 2G,H) alleles. The genetic synergy is more striking between the weak ap1-3 and seu-1. Both seu-1 and ap1-3 single mutants are fertile and develop petals in whorl 2 (Fig. 2A-C). In ap1-3 seu-1 double mutant flowers, whorl 1 organs are transformed into carpelloid organs with horn-like projections and whorl 2 organs are absent (Fig. 2E). The double mutants form small siliques and exhibit reduced fertility. In addition, ap1-3/ap1-3 plants heterozygous for seu-1 (i.e. ap1-3/ap1-3; seu-1/+), exhibited a much stronger floral phenotype than did ap1-3 single mutants (Fig. 2F). In addition to the floral phenotype, plant height is also affected, with ap1-3/ap1-3; seu-1/+ being 75% of the height of ap1-3 single mutants and ap1-3 seu-1 double homozygotes being 50% of the height of ap1-3 single mutants (Fig. 2D). seu-1 single mutants are similar to seu-1 ap1-3 in height. Overall, seu-1 ap1-3 double mutants showed a floral phenotype similar to but more severe than seu-1, suggesting that ap1-3 enhances seu-1 in floral organ identity specification.

By contrast, the ap1-1 seu-1 double mutants (Fig. 2LJ) exhibited a more dramatic enhancement in the meristem defects with inflorescences resembling, but being less severely affected than, those of ap1 cal double mutants. Each floral meristem gave rise to a secondary floral meristem, which generated tertiary and higher order

![Fig. 2. Synergistic genetic interactions between seu and ap1.](image-url)
meristems (Fig. 2J). Eventually, these floral meristems differentiated into flowers with carpelloid first whorl organs (Fig. 2J) and reduced fertility. seu-1/+ heterozygotes also enhanced ap1-1 giving rise to more pronounced meristem defects than did ap1-1 single mutants (data not shown). Our data strongly support a functional reliance of AP1-SEU interaction, not only in organ identity specification, but also in meristem identity specification.

**SEU and LUG modulate transcription activity of AP1 and SEP3 in yeast**

To reveal the molecular mechanism underlying the interaction between AP1/SEP3 and SEU, we tested whether direct interaction between AP1 and SEU, or between SEP3 and SEU, in yeast could lead to the recruitment of LUG and the subsequent repression of reporter gene expression. Yeast containing an integrated GAL7-lacZ reporter was transformed with API-BD or SEP3-BD in the presence or absence of SEU or LUG. API-BD and SEP3-BD were previously reported to activate a lacZ reporter via their C-terminal domain (Honma and Goto, 2001) (Fig. 3A, lanes 3, 9). This activity of API-BD or SEP3-BD was reduced by 50-62% when SEU was induced by galactose (Fig. 3A, lanes 4, 10). This was, at first, unexpected, as SEU-BD was previously shown to exhibit no repressor activity when tethered to heterologous promoters by GAL4-BD (Sridhar et al., 2004). The reduction of lacZ by SEU is likely to be due to a direct physical block of the API and SEP3 C-terminal domain by SEU. lacZ expression was further reduced to background level when LUG was introduced together with SEU (Fig. 3A, lanes 6, 12). In the absence of SEU, LUG did not exert any repressor activity in yeast expressing API-BD or SEP3-BD (Fig. 3A, lanes 5, 11). The requirement for SEU in mediating the effect of LUG was further demonstrated by showing a lack of enhanced repression when LUGΔδLUFS (LUG lacking the LUFS domain) instead of full-length LUG was introduced into yeast expressing API-BD or SEP3-BD together with SEU (Fig. 3A; lanes 8, 14). LUGΔδLUFS could no longer interact with SEU (Sridhar et al., 2004) and was unable to be recruited to repress the reporter gene.

**Transcription activation and repression of AG can be mediated by AP1 and SEP3**

The second intron of AG was previously shown to direct GUS reporter expression in a pattern identical to endogenous AG (Busch et al., 1999; Sieburth and Meyerowitz, 1997). This pAG1::GUS reporter responded to LUG regulation (Sieburth and Meyerowitz, 1997). The AG second intron can be divided into two non-overlapping but functionally redundant 5’ and 3’ enhancers (Busch et al., 1999; Deyholos and Sieburth, 2000). The 3’ enhancer contains the binding sites for LFY and WUSCHEL (WUS), as well as two CArG boxes, the target-binding sites of MADS box proteins. To test API, SEP3, LUG and SEU activity in a biologically relevant context, we constructed a LUC reporter driven by the 3’ enhancer (pAG3::LUC; Fig. 3B).

Because a direct regulatory role of AG by API or SEP3 has not been established, we first tested whether API or SEP3 could regulate pAG3::LUC expression. In a transient assay using onion epidermal cells, pAG3::LUC responded to API and SEP3 activation (Fig. 3C, lanes 2, 6), with SEP3 showing a higher transcriptional activation activity than API1. To test whether LUG and SEU could repress the positive regulatory effect of API and SEP3 on the pAG3::LUC reporter, 35S::SEU or 35S::LUG was co-transfected with 35S::API1 or 35S::SEP3. 35S::LUG alone does not interfere with API or SEP3 activities (Fig. 3C, lanes 3, 7). By contrast, 35S::SEU reduced the LUC expression level to 48-55% (Fig. 3C, lanes 4, 8). Simultaneous introduction of 35S::LUG and 35S::SEU into the onion epidermal cells further reduced reporter expression to about 24-26% (Fig. 3C, lanes 5, 9), suggesting that, through its interaction with SEU, LUG is recruited to the pAG3::LUC reporter to repress its expression. This result suggests that API and SEP3 may act upon the AG 3’ enhancer, possibly via the two CArG boxes. Similar to the results obtained from reporter gene expression in yeast (Fig. 3A), API1 and SEP3 are converted from transcription activators to repressors simply by their interaction with the SEU/LUG co-repressor.

**SEU directly associates with the AG 3’ enhancer in vivo**

Although previous genetic and molecular analyses indicated that LUG and SEU exert their negative regulatory effect on AG via the second intron of AG (Sieburth and Meyerowitz, 1997), our second
DISCUSSION

SEU interacts with the C-terminal domain of AP1 and SEP3

The molecular basis for the combinatorial action of the ABCE genes lies in the assembly of four different MADS box transcription factor complexes in four floral whorls to specify four floral organ types (Theissen and Saedler, 2001). Among the MADS box genes belonging to the A, B, C and E classes, AP1 and SEP3 are unique in that their C-terminal domain possesses strong transcription activation activity (Cho et al., 1999; Honma and Goto, 2001), which may be supplied to the ternary protein complexes. In addition, the C-terminal domains of SEP3 and SQUA, the Antirrhinum ortholog of AP1, were shown to mediate the formation of higher order MADS box protein complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001).

The interaction between SEU and the C-terminal domain of AP1 or SEP3 indicates that AP1 and SEP3 also interact with non-MADS box proteins via their C-terminal domain. This observation suggests an exciting and novel mechanism in which SEU may compete with other MADS box proteins for binding to the C-terminal domain of AP1 and SEP3. Previously, SEU-BD alone was reported to exhibit no repressor activity when tethered to heterologous promoters (Sridhar et al., 2004). The reduced ability of AP1 and SEP3 to activate reporter gene expression in the presence of SEU is consistent with a mechanism in which SEU, by competing for binding to the same C-terminal domain of AP1/SEP3, prevents other transcription co-activators from interacting with AP1/SEP3.

In yeast, the repressor effect of SEU may result from a physical block of the C-terminal domain of AP1 and SEP3 by SEU, rendering the C-terminal domain inaccessible to yeast transcription activation machineries. Therefore, the repressor effect of SEU is distinct from that of LUG, which was dependent on histone deacetylases (Sridhar et al., 2004). The recruitment of LUG by SEU further reduces target gene expression.

**SEU and AP1 may function together to regulate floral meristem identity, as well as floral organ identity**

Mutations in AP1 were previously shown to disturb two successive steps of flower development: flower meristem specification and floral organ identity specification (Bowman et al., 1993; Irish and Sussex, 1990). These two successive functions of AP1 are reflected by the mRNA expression pattern of AP1, which initially is expressed throughout the young floral primordium but later (at stage 3) is only present in the outer two whorls (Mandel et al., 1992). The exclusion of AP1 from the inner two whorls is the result of repression by AG (Gustafson-Brown et al., 1994).

**CAULIFLOWER (CAL)**, which is highly similar to AP1, and LFY, a transcription factor, act together with AP1 to regulate meristem identity (Ferrandiz et al., 2000; Weigel et al., 1992). AP1, CAL and LFY promote floral development not only by positively regulating floral organ identity genes, but also by repressing the expression of another MADS box protein, AGL24, to prevent shoot identity (Yu et al., 2004). Much of the inflorescence characters in lfy, ap1 single and ap1 cal1 double mutants were shown to result from ectopic AGL24 expression, and AGL24 was found to be an immediate target of transcription repression by AP1 (Yu et al., 2004). Our observation that ap1-1 seu-1 double mutants accumulate indeterminate inflorescence meristems similarly to ap1-1 cal1 indicates that SEU may assist AP1 in the repression of AGL24.

Once the floral fate is specified, AP1 is involved in class A activity, specifying sepal and petal identity. The carpelloid floral organs in ap1-1 seu-1 and ap1-3 seu-1 double mutants indicate that AP1 is involved in the negative regulation of AG in the outer two whorls, perhaps by its association with the SEU/LUG co-repressor. Our data indicating a role of AP1 in AG repression are supported by previous genetic studies by Bowman et al. (Bowman et al., 1993), who observed staminoid or carpelloid bracts in whorl 1 at a medial...
position in weak ap1 alleles, and petaloid stamens and stamens in whorl 2 of weak and intermediate ap1 alleles, indicating ectopic AG expression in weak ap1 mutants.

**Dual roles of SEP3 in floral homeotic gene activation and MADS box complex formation**

The SEP1, SEP2 and SEP3 floral organ identity genes were first described as being necessary, albeit redundantly, for the normal development of petals, stamens, and carpels, as triple sep1/2/3 mutants developed flowers with indeterminate whorls of sepals, a phenotype that mirrors the double mutants of B and C genes (Pelaz et al., 2000). Recently, SEP4 has been reported, and sep1/2/3/4 quadruple mutants develop indeterminate flowers with only leaf-like organs (Ditta et al., 2004), indicating that all A, B and C genes require the function of SEP genes. This requirement was reported to be for the formation of multimeric complexes with SEP proteins supplying the transcriptional activation function to the complex (Honma and Goto, 2001).

Two recent studies indicated that SEP3 functions at other levels in addition to being a member of organ-specific MADS box protein complexes. First, AG was found to autoregulate its own transcription (Gomez-Mena et al., 2005), and this positive autoregulation of AG requires the AG/SEP3 complex. Second, 35S::SEP3 transgenic lines resulted in the homeotic transformation of sepals into carpelloid structures (Castillejo et al., 2005). Therefore, AG must be activated ectopically in 35S::SEP3 plants. The same studies also suggested that ectopic SEP3 led to ectopic AP1 expression. Therefore, SEP3 functions both as a component of the organ-specific MADS box protein complex, and as a transcriptional activator of the B and C class genes.

**AP1 and SEP3 may function as both activators and repressors**

Many transcription factors, including MADS box proteins, could function both as activators and repressors depending on their interaction with co-activators or co-repressors. For instance, the MYOCYTE ENHANCER FACTOR-2 (MEF2) MADS box protein is capable of repressing or activating transcription by association with a variety of co-repressors or co-activators in a calcium-dependent manner (Han et al., 2003). Therefore, AP1 may positively regulate organ identity genes such as AG at the early phase of meristem determination and negatively regulate AG at the later phase of organ identity determination by associating with different co-factors. The function of SEP3 both as an activator of AG (in the AG/SEP3 complex) and as a repressor of AG (in the putative SEP3/SEU/LUG complex) may explain why ectopic AG is not observed in sep triple or sep quadruple mutants, because the role of SEP3 in AG activation is epistatic to the role of SEP3 in AG repression.

**A proposed model**

How do LUG and SEU exert their outer whorl-specific repressor effect on AG? We had proposed three alternative models. First, the LUG/SEU complex might interact with DNA-binding partners that are specifically expressed in the outer two whorls of a flower. Second, the LUG/SEU complex may regulate AG indirectly by repressing the expression of a positive regulator of AG. Third, SEU/LUG might repress AG in all four whorls, and some factors in the inner two whorls could antagonize the repressor effect of LUG/SEU.

The finding of AP1 as a DNA-binding partner of SEU/LUG, at the first glance, appears to support the first model. However, several previous observations are not consistent with a role of AP1 in providing an outer whorl-specific repressor activity of AG. First, in situ hybridization did not detect ectopic AG mRNA in strong apr1-1 mutants, and apr1-1 whorl 1 organs are bracts rather than carpels (Gustafson-Brown et al., 1994). Second, 35S:AP1 transgenic plants have normal stamens and carpels (Gustafson-Brown et al., 1994; Mandel and Yanofsky, 1995), suggesting that ectopic AP1 in the inner two whorls did not lead to the repression of AG. Furthermore, in ag mutants, AP1 activity is extended into whorls 3 and 4, but AG mRNA expression remained expressed in the inner two whorls. Additionally, SEP3 is expressed in the inner three whorls and does not fit the criteria for being the outer whorl-specific DNA-binding factor. The direct association between SEU and the AG 3' enhancer (shown in Fig. 4) also helps to eliminate the second model.

Therefore, all previous and current results are consistent with the third model, which is explained in Fig. 5. AP1 and SEP3 (and possibly other SEP genes) can all function redundantly as the DNA-binding partners of SEU/LUG, conferring repressor activity in all four whorls of a flower. Although their repressor effect is enhanced in whorls 1 and 2 by the presence of other outer-specific repressors, including AP2 (Bowman and Meyerowitz, 1991; Chen, 2004; Jofuku et al., 1994) and BLR (Bao et al., 2004), it is dramatically weakened and antagonized in whorls 3 and 4 by inner whorl-specific activators of AG. These inner whorl-specific positive regulatory factors include the combined activities of LFY and WUS (Lenhard et al., 2001), and positive autoregulation by the AG/SEP3 complex (Gomez-Mena et al., 2005). Additionally, the AG/SEP3 complex inhibits AP1 transcription, and an interaction between AG and SEP3 may preclude the SEU/LUG co-repressors from interacting with SEP3. As a result, AG is de-repressed only in whorls 3 and 4. Therefore, we propose that the domain-specific expression of AG is regulated by multiple factors exerting opposite regulatory effects upon AG.

**Fig. 5. A proposed model of how the inner whorl-specific activation of AG is achieved.** In whorls 1 and 2, multiple negative regulatory activities impinge upon the AG cis-regulatory region (such as the AG intron II) to prevent AG transcription. These negative regulatory factors include AP2, BLR and SEU/LUG/SEP1 or SEU/LUG/SEP3. In whorls 3 and 4, multiple positive regulatory factors antagonize the negative effect of SEU/LUG/SEP3 to promote AG transcription. These positive regulatory factors include the combined activities of LFY and WUS, as well as positive autoregulation by AG/SEP3. Additionally, the AG/SEP3 complex inhibits AP1 transcription and an interaction between AG and SEP3 may preclude the SEU/LUG co-repressors from interacting with SEP3. Arrows leading from AP1 or SEP3 to respective LUG/SEU/AP1, LUG/SEU/SEP3 or AG/SEP2 complexes indicate the incorporation of these MADS box proteins into the respective protein complexes.
Transcriptional repression is emerging as a major regulatory mechanism underlying many key developmental and signal pathways in higher plants. For example, the Arabidopsis WUSCHEL (WUS) gene was recently shown to directly interact and recruit transcription co-repressors to repress target genes involved in maintenance of the stem cell pool in the shoot apex (Kieffer et al., 2006). Similar to LUG, these WUS-interacting co-repressors possess an N-terminal LisH domain and C-terminal WD repeats. Therefore, insights gained from our study on transcription repression mechanisms and MADS box protein function are relevant to many areas of plant biology.

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