Polycomb-group proteins repress the floral activator AGL19 in the FLC-independent vernalization pathway

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Polycomb-group (PcG) proteins form a cellular memory by maintaining developmental regulators in a transcriptionally repressed state. We identified a novel flowering gene that is under PcG control in Arabidopsis—the MADS-box gene AGL19. AGL19 expression is maintained at very low levels by the PcG proteins MSI1, CLF, and EMF2, and AGL19 is partly responsible for the early flowering phenotype of clf mutants. AGL19 chromatin is strongly enriched in trimethylation of Lys 27 on histone H3 (H3K27me3) but not in H3K9me2. Repressive H3K27me3 marks were reduced by decreased CLF or MSI1 levels and by prolonged cold, suggesting that the PcG proteins MSI1 and CLF repress AGL19 in the absence of cold. Ectopic expression of AGL19 strongly accelerates flowering, and agl19 mutants have a decreased response to vernalization, the promotion of flowering by prolonged cold. Epistasis analyses revealed that AGL19 works in the poorly characterized FLC-independent vernalization pathway and does not require SOC1 to function. In this pathway, prolonged cold relieves AGL19 from PcG repression by a mechanism that requires VIN3 but not VRN2. Elevated AGL19 levels activate LFY and AP1 and eventually cause flowering.

[Keywords: Polycomb proteins; histone methylation; MSI1; vernalization; FLC; SOC1]

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Polycomb-group [PcG] proteins are cellular memory modules that are thought to maintain genes in a transcriptionally inactive state after an initial repression has been established (for review, see Brock and Fisher 2005). PcG proteins were first identified in insects, but are essential for normal development in both animals and plants (for review, see Schubert et al. 2005). These proteins often form large multiprotein complexes, such as the Polycomb Repressive Complex 2 [PRC2]. Drosophila PRC2 contains four core subunits: the MSI1-like protein p55, Enhancer of Zeste [E(Z)], Extra Sex Combs [ESC], and Suppressor of Zeste 12 [Su(Z)12] (Czermin et al. 2002; Müller et al. 2002). Transcriptional repression by PRC2 relies on histone methyltransferase activity of the E(Z) subunit, which preferentially catalyzes histone H3 Lys 27 [H3K27] trimethylation (for review, see Cao and Zhang 2004).

Unlike in insects, where the PRC2 members are encoded by single-copy genes, in plants homologous PRC2 subunits are often encoded by small gene families [Schubert et al. 2005], and several possible PRC2-like complexes have been proposed to exist in Arabidopsis (Chanvivattana et al. 2004; Hennig et al. 2005). However, to date, only one Arabidopsis PRC2 complex has been well characterized—the FERTILISATION INDEPENDENT SEED [FIS] complex (Köhler et al. 2003a). The FIS complex consists of MSI1, the E(Z) homolog MEDEA [MEA], the ESC homolog FERTILISATION INDEPENDENT ENDOSPERM [FIE], and likely the Su(Z)12 homolog FIS2 [Köhler et al. 2003a; Chanvivattana et al. 2004]. This complex has specific functions during gametophyte and early seed development, including suppression of seed development in the absence of fertilization and repression of the MADS-box gene PHERES1 [PHE1] (Chaudhury et al. 1997; Grossniklaus et al. 1998; Ohad et al. 1999; Köhler et al. 2003b). During sporophyte development, a second PRC2 complex, the CURLY LEAF [CLF] complex, most likely represses transcription of floral homeotic genes, such as the MADS-box gene AGAMOUS [AG]. The CLF complex probably consists of MSI1, the E(Z) homolog CLF, FIE, and the Su(Z)12 homolog EMBRYONIC FLOWER2 [EMF2] (Goodrich et al. 1997; Kinoshita et al. 2001; Yoshida et al. 2001; Hennig et al. 2003; Chanvivattana et al. 2004; Katz et al. 2004).
The third potential PRC2-like complex is the VERNALIZATION (VRN) complex. The existence of the VRN complex was hypothesized because the Su(Z)12 homolog VRN2 is required for maintaining repression of the MADS-box gene FLOWERING LOCUS C (FLC) after vernalization and for vernalization-induced H3 methylation at the FLC locus (Chandler et al. 1996; Gendall et al. 2001; Bastow et al. 2004; Chanivivattana et al. 2004; Sung and Amasino 2004). Because FLC is a very potent repressor of flowering, epigenetic control of the floral transition has recently received considerable attention [for review, see He and Amasino 2005].

The transition to flowering is controlled by diverse environmental and developmental signals, and many genes that control flowering in Arabidopsis have been identified [for review, see Boss et al. 2004; Ausin et al. 2005]. These genes have been grouped in several genetic pathways, including the photoperiod, the vernalization, and the autonomous pathways. The photoperiod pathway responds to seasonal changes in day length, while the vernalization pathway renders Arabidopsis competent to flower after prolonged exposure to low temperatures; for example, during winter [for review, see Henderson and Dean 2004; Searle and Coupland 2004]. These two pathways contribute to initiate flowering of winter-annual plants in the favorable conditions of spring and summer. In contrast, the autonomous pathway promotes flowering independently of environmental signals. Both the autonomous and vernalization pathways function mainly by reducing expression of the floral repressor FLC [Michaels and Amasino 1999, 2001; Sheldon et al. 2000; Gendall et al. 2001]. Repression of FLC by vernalization is initiated by VERNALIZATION INSENSITIVE3 (VIN3) and maintained by VERNALIZATION1 (VRN1) and VERNALIZATION2 (VRN2) [Gendall et al. 2001; Levy et al. 2002; Sung and Amasino 2004]. When expressed, FLC prevents transcription of the pathway integrators FLOWERING LOCUS T (FT) and the MADS-box gene SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1, or AGL20) [Borner et al. 2000; Lee et al. 2000; Michaels and Amasino 2001], which combine signals from several pathways to induce flowering [for review, see Parcy 2005]. Vernalization releases SOC1 from FLC repression, thus making SOC1 an important component of the FLC-dependent vernalization pathway [Lee et al. 2000; Hepworth et al. 2002, Moon et al. 2003a]. Although FLC plays a central role in vernalization in Arabidopsis, flc-null mutants still respond to vernalization, demonstrating that FLC-independent mechanisms exist in the vernalization pathway [Michaels and Amasino 2001; Sung and Amasino 2004]. However, the molecular nature of these FLC-independent mechanisms is poorly understood.

Among the Arabidopsis homologs of PRC2 subunits, the floral transition is not only regulated by the Su(Z)12 homolog VRN2, but also by the p55 homolog MSI4 [also called FVE] [Koonmeen et al. 1991; Ausin et al. 2004; Kim et al. 2004]. FVE is part of the autonomous pathway, needed for the epigenetic repression of FLC via histone deacetylation. In addition to FVE/MSI4, Arabidopsis contains four other MSI1-like proteins [MSI1–3, MSI5] [Ach et al. 1997; Hennig et al. 2003]. Although MSI1-like proteins have no catalytic activity, they are often essential subunits of protein complexes controlling chromatin dynamics [for review, see Hennig et al. 2005]. Arabidopsis MSI1, for instance, is vital for plant development, as msi1-null mutants are embryo lethal [Köhler et al. 2003a; Guitton et al. 2004]. Strong reduction of MSI1 protein levels by cosuppression [msi1-cs plants] is not lethal but has severe impacts on development [Hennig et al. 2003]. In this study, we performed transcriptional profiling of msi1-cs plants and found that the MADS-box gene AGL19 is under control of the MSI1, CLF, and EMF2 Arabidopsis PcG proteins. AGL19 is a potent floral activator, which is repressed via H3K27 trimethylation in the absence of vernalization. This work also establishes a novel role for PcG proteins as repressors of the FLC-independent vernalization pathway of Arabidopsis.

**Results**

AGL19 is up-regulated in msi1-cs plants

In order to identify genes affected by the severe reduction in MSI1 levels in msi1-cs plants, we performed microarray RNA profiling experiments on rosette leaves of 23-d-old wild-type and msi1-cs plants grown in long days (LD). This developmental stage was chosen because phenotypic alterations of msi1-cs rosette leaves can then first be observed [Hennig et al. 2003]. Statistical analysis of the microarray data identified eight robustly down-regulated genes and 122 robustly up-regulated genes. These deregulated genes fall into diverse categories, with a large proportion being involved in histone metabolism, cell cycle, and DNA repair. The MADS-box gene AGL19 was among the strongest up-regulated genes, with a 21-fold increase in msi1-cs leaves, and was the only MADS-box gene with a significantly altered expression pattern [Fig. 1A]. RT–PCR confirmed that AGL19 is usually expressed at very low levels in wild-type rosette leaves but was strongly up-regulated in msi1-cs leaves [Fig. 1B]. AGL19 belongs to the type II class of MADS-box genes and is phylogenetically most closely related to AGL14 and to the floral pathway integrator SOC1/AGL20 [Becker and Theissen 2003]. So far, AGL19 has not been extensively characterized, and its biological function is unknown. Because MADS-box transcription factors are often developmental key regulators, AGL19 was selected for a detailed study.

AGL19 is a weakly expressed transcription factor localized in the nucleus

AGL19 was originally considered as a root-specific gene [Alvarez-Buylla et al. 2000]. However, as we detected AGL19 in wild-type rosette leaves using both microarrays and RT–PCR, we re-examined the expression pattern of AGL19 in different organs and compared it with
more detailed analysis of the *AGL19* expression pattern in the apex by in situ hybridization. Localization studies using GFP fusion proteins showed that *AGL19* is targeted to the nucleus (Fig. 1G). Together, *AGL19*, but not its close homolog *AGL14*, is expressed with a similar organ-specificity as the well-known floral integrator *SOC1*.

**AGL19 is sufficient to promote flowering**

To study the effects of ectopic *AGL19* expression, we constructed transgenic plants containing the *AGL19* cDNA under the control of the strong cauliflower mosaic virus 35S promoter (35S:*AGL19*) (Fig. 2A). Five independent transgenic lines were analyzed, all of which were extremely early flowering in LD conditions and produced two to four rosette leaves at the time of bolting (Fig. 2B; Table 1). In noninductive SD conditions, 35S:*AGL19* plants were also early flowering but produced more leaves than in LD, and therefore, they remained responsive to photoperiod (Fig. 2C). In the late flowering fve background, the overexpression of *AGL19* had the strongest effect on flowering time as 35S:*AGL19* fve plants flowered similarly to 35S:*AGL19* plants (Table 1). Thus, overexpression of *AGL19* is sufficient to promote flowering.

**agl19 mutants have a decreased vernalization response**

To determine whether *AGL19* normally controls flowering time in wild-type plants, we used two *agl19* insertion alleles (*agl19-1* and *agl19-2*), in which the *AGL19* transcript was undetectable (Fig. 3A,B). The *agl19* mutants flowered normally in LD conditions, but flowered slightly later than wild type in SD (Fig. 3C). Because overexpression of *AGL19* caused phenotypes similar to overexpression of *SOC1* (Borner et al. 2000), which is regulated by the vernalization pathway, we tested whether *AGL19* was also involved in the response to vernalization. Indeed, *agl19* mutants considered...
AGL19 is dispensable for the establishment and maintenance of FLC repression [Fig. 4G]. Taken together, these results show that AGL19 is not repressed by FLC in wild-type plants and that the reduced vernalization response in agl19 mutants is not caused by elevated FLC levels. Thus, it is possible that AGL19 functions in an FLC-independent branch of the vernalization pathway.

In order to genetically test whether AGL19 functions in the FLC-independent vernalization pathway, the vernalization response of the agl19 flc double mutant was tested. This double mutant responded less to vernalization than either single mutant [Table 2]. Thus, the agl19 and flc mutants exhibit an additive rather than an epistatic relationship, confirming that AGL19 functions in an FLC-independent vernalization pathway. Similar to some previous reports [e.g., Zhang and van Nocker 2002], the flc mutant flowered later than wild type in SD after vernalization in several independent experiments. The reasons for this effect are not known, but indirect effects mediated by poorly understood FLC targets and functions [McKay et al. 2003; Edwards et al. 2006] might be involved.

### Table 1. Effect of 3SS::AGL19 on flowering time in LD

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Leaf number at bolting a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>11.6 ± 0.5</td>
</tr>
<tr>
<td>3SS::AGL19a</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>3SS::AGL19b</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>3SS::AGL19c</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>3SS::AGL19d</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>3SS::AGL19e</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Wild type</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>fve</td>
<td>26.6 ± 1.9</td>
</tr>
<tr>
<td>3SS::AGL19a</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>fve 3SS::AGL19a</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

Note that two independent flowering time experiments are separated by an empty line.

aMean ± S.D.; n ≥ 14.

### AGL19 function does not require SOC1

The FLC-dependent vernalization pathway functions mainly to control expression of the floral integrator SOC1 [Lee et al. 2000; Gendall et al. 2001; Hepworth et al. 2002; Levy et al. 2002; Moon et al. 2003a, 2005]. Therefore, we next investigated whether the AGL19-
which is induced by ectopic SOC1 expression [Michaels et al. 2003]. Because ectopic overexpression of SOC1 strongly promotes flowering without increasing AGL19 levels, SOC1 does not function via activation of AGL19 expression. Moreover, because ectopic overexpression of AGL19 strongly promotes flowering without increasing SOC1 levels, AGL19 does not function via activation of SOC1 expression. Rather, it is possible that SOC1 and AGL19 function in genetic parallel but cross-connected pathways.

To examine whether AGL19 and SOC1 function in the same or in parallel genetic pathways to promote flowering, the agl19 soc1 double mutant was generated. If AGL19 and SOC1 act in the same pathway, then the late-flowering phenotype of the double mutant should not transgress that of the latest single mutant. However, the agl19 soc1 double mutant flowered later than the soc1 single mutant, and this delay was more pronounced in SD [additional 15 leaves] than in LD [additional two leaves] [Fig. 4H,I], indicating that AGL19 and SOC1 act in genetically different flowering pathways. Similarly, 35S::AGL19 soc1 plants were still early flowering, but showed an additive rather than an epistatic phenotype, thus confirming that AGL19 and SOC1 probably function in parallel pathways to promote flowering [Fig. 4H,I]. Nonetheless, it is possible that AGL19 and SOC1 regulate common downstream targets. Indeed, expression of the floral integrator LEAFY (LFY) and of the meristem identity gene APETALA1 [API] was increased in 35S::AGL19 and 35S::SOC1 seedlings [Supplementary Fig. S1].

Control of AGL19 by Polycomb-group complexes

As AGL19 expression was increased in msi1-cs plants, it may be repressed by a MS1-containing Arabidopsis PROC2 complex, like the CLF–PROC2 complex, which functions during sporophytic development [Schubert et al. 2005]. Indeed, AGL19 was similarly up-regulated in 15-d-old emf2 and clf seedlings as well as in 4-wk-old rosette leaves of clf and msi1-cs plants [Figs. 5A, 6A]. Interestingly, derepression of AGL19 in clf increased with plant age [Fig. 5B]. These results show that MS1, CLF, and EMF2, three likely subunits of the CLF complex, are all required to repress AGL19 in the absence of a vernalization treatment. As AGL19 shares similarities with SOC1, we tested whether CLF represses SOC1 as well. In contrast to AGL19, SOC1 expression was not

Table 2. Effect of vernalization on flowering time of agl19 and flc mutants in SD

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Leaf number−vernualization</th>
<th>Leaf number+vernalization</th>
<th>Leaf difference ± vernalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>58.1 ± 3.8</td>
<td>27.6 ± 3.2</td>
<td>30.5</td>
</tr>
<tr>
<td>agl19</td>
<td>59.7 ± 3.9</td>
<td>43.3 ± 2.4</td>
<td>16.4</td>
</tr>
<tr>
<td>flc</td>
<td>51.9 ± 2.3</td>
<td>33.1 ± 3.8</td>
<td>18.8</td>
</tr>
<tr>
<td>agl19 flc</td>
<td>55.2 ± 4.7</td>
<td>46.3 ± 1.7</td>
<td>8.9</td>
</tr>
</tbody>
</table>

*Mean ± S.D.; n ≥ 14.
Figure 5. AGL19 is up-regulated in PcG mutants and contributes to the early flowering phenotype of clf. (A) Expression of AGL19 in clf and emf2. RNA was extracted from 15-d-old seedlings grown in LD. (B) Age dependency of AGL19 derepression in clf. RNA was extracted at 10, 15, and 33 d after germination. (C) Expression of SOC1 in clf. RNA was extracted from 10-d-old seedlings and from rosette leaves of 21-d-old plants grown in LD. (D) CLF, EMF2, and MSI1 expression in 10-d-old wild-type [WT] seedlings grown in SD with or without a 6-wk vernalization treatment (Vern). [E] ChiP using anti-myc antibodies and 10-d-old wild-type Landsberg erecta or transgenic 35S::myc-FIE seedlings grown in LD. Shown are PCR products of fragments II and III from the AGL19 locus (see Fig. 6 for details) and of an unrelated PHOSPHOFRUCTOKINASE [PFK] gene. (F) Flowering time of msi1-cs plants in SD. (G) Flowering time of wild-type [WT], agl19, clf, and clf agl19 plants in LD. Leaf numbers differ significantly between clf and clf agl19 double mutants (t-test: p < 0.001). (H) Images of 22-d-old wild-type [WT], agl19, clf, and clf agl19 plants grown in LD at 21°C. Note that all clf mutants are already flowering, while agl19 clf double mutants have only just started bolting.

Consistent with the findings that AGL19 can promote flowering and that AGL19 expression is increased in msi1-cs plants, msi1-cs plants flowered significantly earlier than wild type in SD [Fig. 5F]. Because cosuppression of MSI1 occurs around an age of 2–3 wk in LD, it cannot affect flowering time any more at this stage, and msi1-cs plants were not consistently earlier flowering than wild type in LD [data not shown]. Similarly, AGL19 expression is increased in clf and emf2 mutants, and both flower earlier than wild type (Yang et al. 1995; Goodrich et al. 1997), suggesting that the overexpression of AGL19 observed in these mutants could be partially responsible for their early flowering phenotype. In order to test this hypothesis, we constructed the clf agl19 double mutant. Loss of AGL19 did not abolish the clf leaf phenotype, but partially suppressed the early flowering phenotype [Fig. 5G,H]. Thus, elevated AGL19 levels specifically contribute to the early flowering of clf.

MSI1 and CLF epigenetically regulate the AGL19 locus

In animals, the E(Z)-related subunit of PRC2-like complexes has H3K27 methyltransferase activity [Cao and...
AGL19 has been identified as a novel floral activator in Arabidopsis, because overexpression of AGL19 greatly accelerates the transition to flowering, while loss of AGL19 delayed flowering after a vernalization treatment. AGL19 belongs to the MADS-box gene family, which contains master regulatory genes essential for diverse developmental processes (Becker and Theissen 2003). Plant MADS-box genes form several phylogenetically distinct groups, and AGL19 together with its closest homolog AGL14 (68% amino acid identity) and the well-characterized floral activator SOC1 (AGL20, 46% amino acid identity) belong to the Tm3/SOC1 clade (Becker and Theissen 2003). Although AGL19, AGL14, and AGL20 are closely related, their expression patterns differ. In particular, AGL14 is exclusively expressed in roots, while AGL19 and SOC1 are expressed in many organs of the shoot as well. Despite the prominent expression of AGL19 in roots, root growth and macroscopic root morphology did not differ between wild-type, agl19, and 35S::AGL19 plants (Supplementary Fig. S2, data not shown).

AGL19 and SOC1 both function to promote flowering, but they have diverged significantly in terms of gene regulation and flowering pathways in which they participate. The agl19 mutant flowered normally in LD and was only slightly late flowering in SD, indicating that under standard laboratory conditions, AGL19 has only a minor role in the promotion of flowering. In contrast, the soc1 mutant is significantly late flowering in both LD and SD (Borner et al. 2000; Lee et al. 2000). Because agl19 mutants could no longer adequately accelerate flowering in response to prolonged cold treatments, AGL19 functions in the vernalization response. While SOC1 integrates several genetic pathways that promote flowering, including the vernalization and photoperiod pathways (Borner et al. 2000; Lee et al. 2000; Samach et al. 2000; Hepworth et al. 2002; Moon et al. 2003a), AGL19 is mainly part of the vernalization pathway.

Similarly to ectopic expression of SOC1, ectopic expression of AGL19 did not only greatly accelerate flowering but caused defects in floral development as well (Borner et al. 2000; data not shown). The fact that only 35S::AGL19 but not agl19 plants had altered flower morphology suggests that like SOC1, AGL19 is also dispensable for flower development and appears to function specifically during the floral transition after vernalization.

AGL19 regulates the floral transition in an FLC-independent vernalization pathway

Vernalization in Arabidopsis acts mainly via repression of FLC and subsequent up-regulation of SOC1 (Lee et al. 2000; Gendall et al. 2001; Hepworth et al. 2002; Levy et al. 2002; Moon et al. 2003a). However, flc-null mutants still show a vernalization response, and thus an FLC-
independent vernalization pathway must exist [Michaels and Amasino 2001]. Although both AGL19 and SOC1 are up-regulated by vernalization, only SOC1 but not AGL19 is repressed by FLC. In addition, AGL19 levels had no effect on the amount of FLC transcript, and FLC remained repressed after vernalization in the agl19 mutant. Thus, AGL19 does not regulate FLC, and the reduced vernalization response of agl19 mutants is not caused by deregulation of FLC as in the vrn1 and vrn2 mutants. Because agl19 flc double mutants had an additive effect on the vernalization response, AGL19 and FLC function in genetically separate pathways. The FLC-containing pathway requires VIN3 and VRN2 [Gendall et al. 2001, Sung and Amasino 2004]. The AGL19-containing pathway requires VIN3 but not VRN2. Both elevated AGL19 and SOC1 levels lead to the activation of LFY and AP1 to promote flowering.

Interestingly, even the agl19 flc double mutant showed a residual vernalization response, indicating the presence of other vernalization pathways. Such a pathway might include the MADS-box gene AGL24, which is known to mediate an FLC-independent vernalization response [Yu et al. 2002; Michaels et al. 2003]. Analysis of the vernalization response of agl19 ffc agl24 triple mutants will help answer this question.

Although AGL24 acts in an FLC-independent pathway, it is closely linked to SOC1, because these two genes positively regulate each other’s expression, and overexpression of one of the two genes has little effect in the absence of the other [Michaels et al. 2003]. In contrast, AGL19 and SOC1 act at least partially independently, because there seems to be no or even negative cross-regulation between AGL19 and SOC1 and overexpression of AGL19 greatly accelerated flowering in a soc1 background. Both agl19 soc1 as well as 35S::AGL19 soc1 showed additive rather than epistatic interactions, indicating that SOC1 and AGL19 act in separate genetic pathways that converge to control the common downstream targets AP1 and LFY. Thus, although SOC1, AGL24, and AGL19 share several similarities, they clearly have different roles in the genetic network controlling flowering, and are all needed to coordinate a fully functional vernalization response.

AGL19 repression depends on PRC2 subunits

In the absence of vernalization, AGL19 is lowly expressed, and MSII, CLF, and EMF2 are all required for repression of AGL19. MSII, CLF, and EMF2 are homologs of Drosophila PRC2 subunits [Ach et al. 1997; Goodrich et al. 1997; Yoshida et al. 2001], and it was suggested that these proteins together with FIE form an Arabidopsis PRC2 complex [Chanvivattana et al. 2004]. Protein–protein interaction data support this model as: MSII interacts with FIE, FIE interacts with CLF, and CLF interacts with EMF2 [Köhler et al. 2003a; Chanvivattana et al. 2004; Katz et al. 2004]. ChiP experiments suggested that AGL19 is a direct target gene of the CLF–PRC2 complex and established that AGL19 chromatin is enriched in H3K27me3 marks, which at least in mammals and insects are set by PRC2 complexes [for review, see Chiang and Zhang 2004]. Arabidopsis has several homologs of PRC2 subunits and probably contains several PRC2-like complexes, which may function in different tissues or at different developmental stages [for review, see Schubert et al. 2005]. PRC2-like complexes are thought to function as transcriptional repressors, and other Arabidopsis PcG target genes include PHERES1 (PHE1), AGAMOUS (AG), and FLC. MEA, FIE, and FIS2 are required to repress PHE1 [Köhler et al. 2003b], MSII, CLF, FIE, and EMF2 are required to repress AG [Chen et al. 1997; Goodrich et al. 1997; Kinoshita et al. 2001; Hennig et al. 2003], and VRN2 is required to repress FLC [Gendall et al. 2001]. Interestingly, PHE1, AG, and FLC as well as AGL19 are all MADS-box genes, supporting the notion that in contrast to metazoan PRC2 complexes, which mostly control Homeobox genes, plant PRC2 complexes often control MADS-box transcription factors. Importantly, only AGL19, but not SOC1 or AG, needs MSII, CLF, and EMF2 to remain repressed in the absence of vernalization [this study; cf. also microarray data in Moon et al. 2003b]. This suggests that either PRC2 repression was a feature of the Tm3/SOC1 ancestor and was lost in SOC1 and AGL14, or that AGL19 acquired PRC2 repression de novo. However, plant PRC2 complexes also have non-MADS-box targets [Katz et al. 2004], and more complete lists of primary and secondary plant and metazoan PRC2 target genes are needed for more systematic comparisons.

AGL19, which is a strong activator of flowering, is up-regulated in PRC2 mutants, and clf, fie, emf2, and msi1-cs plants are all early flowering. This suggests that CLF, EMF2, MSI1, and FIE form a PRC2 complex to control flowering time. MSI1 not only inhibits flowering by contributing to normal SOC1 expression, this function, however, is independent of CLF [Bouveret et al. 2006]. The contribution of increased AGL19 expression to the early flowering phenotype of msi1-cs plants could not be tested due to silencing of the 35S::MSII transgene by the AGL19 SALK-insertion allele in the 35S::MSII agl19 plants [data not shown]. The 35S::MSII transgene is required to induce MSII cosuppression. In clf mutants, we found that AGL19 was partially responsible for the early flowering phenotype, as the agl19 clf double mutant flowered later than the clf mutant alone. However, repression of AGL19 is not the major cause of the clf phenotype, and this is consistent with previous reports of a prominent role of ectopic AG expression for the clf phenotype [Goodrich et al. 1997]. In clf mutants, expression of AG is correctly initiated in young floral meristems, but later breaks down and becomes expressed in the outer floral whorls [Goodrich et al. 1997]. Similarly, PHE1 is initially normally expressed in fis mutants but is subsequently ectopically expressed in the endosperm [Köhler et al. 2003b], and FLC is initially repressed by vernalization treatments but subsequently becomes derepressed in vrn2 mutants [Gendall et al. 2001]. Interestingly, the pattern of progressive AGL19 derepression in clf mutants is strikingly similar to that of other PcG
targets in plants and Drosophila, where target genes are initially expressed normally but subsequently become ectopically activated (for review, see Ringrose and Paro 2004; Brock and Fisher 2005).

H3K27 trimethylation at AGL19 chromatin depends on PRC2 subunits and is regulated by vernalization

The mechanism of transcriptional repression by PRC2 complexes is not yet completely understood, but trimethylation of H3K27 appears to be of central importance in vivo (for review, see Cao and Zhang 2004). In Arabidopsis, H3K27me3 marks are found mostly in euchromatin [Lindroth et al. 2004], while H3K9me2 marks are hallmarks of silent heterochromatic genes [Soppe et al. 2002]. Because AGL19 is only enriched in H3K27me3 but not in H3K9me2, repression of AGL19 is probably different from heterochromatic silencing as observed for many transposons. Instead, AGL19 appears to be a euchromatic gene that is repressed by H3K27me3. Interestingly, vernalization reduced H3K27me3 marks to lower levels than either reductions of MSI1 or loss of CLF. In Arabidopsis, H3K27me3 marks are hallmarks of silent heterochromatic genes (Lindroth et al. 2004), while H3K9me2 marks are hallmarks of silent heterochromatic genes [Soppe et al. 2002]. Because AGL19 is only enriched in H3K27me3 but not in H3K9me2, repression of AGL19 is probably different from heterochromatic silencing as observed for many transposons. Instead, AGL19 appears to be a euchromatic gene that is repressed by H3K27me3. Similarly, partial redundancy of CLF and its homolog SWN has been observed [Chanvivattana et al. 2004]. Thus, it is possible that functional redundancies attenuate the effects on AGL19 chromatin modification in msi1-cs and clf.

H3K27me3 was not spread uniformly across the AGL19 locus but peaked dramatically near the transcription start site. Similarly, binding of Drosophila Polycomb proteins seems to cluster at Polycomb-responsive elements (PREs) and promoters rather than being spread uniformly over target loci [Ringrose and Paro 2004; Brock and Fisher 2005]. Consistent with this local clustering, Polycomb silencing blocks transcription initiation in Drosophila [Dellino et al. 2004]. In Arabidopsis, the PIS PRC2 complex was found to bind preferentially at the PHE1 promoter [Köhler et al. 2003b], and FLC chromatin is enriched in H3 methylation at the promoter and the first large intron [Bastow et al. 2004; Sung and Amasino 2004]. Taken together, these results suggest that AGL19 is repressed by a PRC2 complex containing MSI1, CLF, FIE, and EMF2 that sets H3K27me3 marks close to the transcriptional start site, similar to the situation in other Polycomb target genes.

Vernalization overcomes the repressive effect of the CLF–PRC2 complex. This could be carried out by active removal of the repressive H3K27me3 marks at AGL19. Because no biochemical activity that removes trimethylation is known, it is more likely that vernalization prevents the establishment and/or maintenance of H3K27me3 by the CLF–PRC2 complex during cell proliferation. This interpretation is supported by observations that vernalization often acts on dividing cells such as those present in meristems (for review, see Ausin et al. 2005). An alternative mechanism could involve exchange of methylated histones in AGL19 chromatin.

Together, the vernalization pathway in Arabidopsis contains at least two branches—the FLC-dependent and the FLC-independent branches, both of which require the regulatory effects of Polycomb proteins. While VRN2 functions in repressing the floral repressor FLC after vernalization, the CLF complex functions in repressing the floral activator AGL19 before vernalization. Thus, in Arabidopsis, different Polycomb proteins have been recruited to coordinate the vernalization response and to control the important developmental switch to reproductive growth.

Materials and methods

Plant material and growth conditions

Line msi1OEc2, which ectopically expresses MSI1 and gives rise to msi1-cs plants, was described before [Henning et al. 2003]. To construct plants expressing an epitope-tagged version of FIE, a construct encoding an N-terminal fusion of myc to FIE was cloned under the control of the 35S promoter and transformed into wild-type Ler plants. Line 4, which expressed the myc-FIE fusion [Supplementary Fig. S1], was used for further experiments. The two null AGL19 mutant alleles, agl19-1 [SALK_N578786] and agl19-2 [SALK_N516657] were obtained from the Nottingham Arabidopsis Seed Stock Center (NASC). The agl19-1 allele was used for all crossings and flowering time experiments unless otherwise stated. The fve-5, fce-6, vrn1-5, and clf-29 mutants are null alleles [Supplementary Fig. S1; Bouvier et al. 2006] from various collections of T-DNA insertion lines [SAIL_1167E5, SALK_41126, WiscDsLox393-396E9, and SALK_N521003, respectively] [Sessions et al. 2002; Alonso et al. 2003]. All plants used in this study are in the Columbia background unless otherwise stated. Seeds of soc1-2, emf2-10, and emf2-1 have been described [Chandler et al. 1996; Lee et al. 2000; Chanvivattana et al. 2004] and were kindly provided by I. Lee [Seoul National University, Seoul, Korea], J. Goodrich [University of Edinburgh, Edinburgh, UK], and C. Dean [John Innes Centre, Norwich, UK], respectively.

To construct plants that ectopically overexpressed AGL19 or SOC1 [35S::AGL19 and 35S::SOC1], the full-length coding sequences were amplified by PCR (for primers, see Supplementary Table S1). The cDNAs were inserted into binary destination vectors [AGL19 in pH7WG2; SOC1 in pK7WG2] [Karimi et al. 2002] downstream of the cauliflower mosaic virus [CaMV] 35S promoter. Constructs were transformed into Columbia wild-type plants, and 43 primary 35S::AGL19 transformants were obtained, of which five [35S::AGL19a–e] were selected for further study. The weak line 35S::AGL19a was used for all crossings and flowering time experiments unless otherwise stated.

For cellular localization studies, the full-length AGL19 cDNA was fused to an N-terminal GFP tag in vector pK7WG2 [Karimi et al. 2002]. AGL19_pK7WGF2 was used directly for bombardment of onion cells for transient expression assays as described previously [Gendall et al. 2001].

For measuring flowering time, seeds were plated on Murashige and Skoog [MS] medium [Duchefa], stratified for 2 d at 4°C, and grown on plates for 10 d before transfer onto soil. Plants were kept in Conviron growth chambers with mixed cold fluorescent and incandescent light [110–140 μmol/m²·sec, 21°C ± 2°C] under LD [16 h light] photoperiods unless indicated otherwise. The flowering time was measured as the number of total rosette leaves longer than 0.5 cm at bolting for at least 14 plants. Graphs show means ± standard deviation. For the vernalization treatment, seeds were plated on MS medium and kept in continuous light for 1 d before being exposed to 4°C for growth.
6 wk. After the vernalization treatment, plants were transferred to growth chambers [21°C ± 2°C] under LD or SD (8 h light) photoperiods as indicated.

**RNA isolation and RT–PCR**

RNA was extracted as previously described [Hennig et al. 2003]. DNA-free RNA was reverse-transcribed using an oligo[dT] primer and SuperScript II reverse transcriptase [Invitrogen]. Aliquots of the generated cDNA, which equaled 100 ng of total RNA, were used as the template for PCR with gene-specific primers [Supplementary Tables S2, S3]. Q-PCR was performed in an ABI Prism 7700 Sequence Detection System [Applied Biosystems], using SYBR Green Master Mix reagent [Applied Biosystems] according to the manufacturer’s instructions. All amplification plots were analyzed with a fluorescence signal threshold of 0.1 to obtain Ct [threshold cycle] values. Experiments were performed in duplicate with error bars representing the range. Gene expression levels were normalized to an actin [ACT] control gene. The average Ct value for ACT was 23.83 ±0.18 for all templates measured in these experiments.

**Microarray hybridization and evaluation**

*Arabidopsis* wild-type (accession Columbia) and msi1OEc2 plants [Hennig et al. 2003] were grown for 23 d in growth chambers at 21°C under LD photoperiods. After identification of msi1-cs plants from the segregating msi1OEc2 population based on phenotype, rosette leaves were harvested. Material was harvested from at least 12 plants and pooled for each sample. The experiment was repeated twice to give two independent biological replicates. Affymetrix *Arabidopsis* ATH1 GeneChips were used in the experiment [Affymetrix]. Labeling of samples, hybridizations, and measurements were performed as described [Hennig et al. 2004]. Signal values were derived using the GCRMA algorithm in the statistical package R [version 1.9.1]. Significantly differential gene expression was detected based on the rank-product algorithm [Breitling et al. 2004]. This algorithm inherently corrects for multiple testing. Genes were considered as differentially expressed if \( p < 0.05 \).

**ChIP**

ChIP was performed as previously described [Bowler et al. 2004]. Anti-myc 9E11 antibodies [Eurogentec], anti-dimethyl-histone H3 Lys 9 antiserum [Upstate, catalog #07-441], and anti-tri-methyl-histone H3 Lys 27 antiserum [Upstate, catalog #07-449] were used for immunoprecipitation.

Immunoprecipitated DNA was analyzed by PCR using gene specific primers for AGL19 and primers against the control gene *PFK* [At4g04040] [Supplementary Table S4]. Relative efficiencies of primer pairs were determined based on dilution series of input DNA [Supplementary Fig. S3B]. Band intensities were quantified using Image J software. For quantitative displays of ChIP experiments, the values shown represent enrichment relative to *PFK* using the previously determined primer efficiencies. Bars represent mean and range for two independent ChIP experiments.

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**References**


Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Som-


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