Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27

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The plant Polycomb-group (Pc-G) protein CURLY LEAF (CLF) is required to repress targets such as *AGAMOUS* (*AG*) and *SHOOTMERISTEMLESS* (*STM*). Using chromatin immunoprecipitation, we identify AG and STM as direct targets for CLF and show that they carry a characteristic epigenetic signature of dispersed histone H3 lysine 27 trimethylation (H3K27me3) and localised H3K27me2 methylation. H3K27 methylation is present throughout leaf development and consistent with this, CLF is required persistently to silence AG. However, CLF is not itself an epigenetic mark as it is lost during mitosis. We suggest a model in which Pc-G proteins are recruited to localised regions of targets and then mediate dispersed H3K27me3. Analysis of transgenes carrying AG regulatory sequences confirms that H3K27me3 can spread to novel sequences in a CLF-dependent manner and further shows that H3K27me3 methylation is not sufficient for silencing of targets. We suggest that the spread of H3K27me3 contributes to the mitotic heritability of Pc-G silencing, and that the loss of silencing caused by transposon insertions at plant Pc-G targets reflects impaired spreading.

Introduction

In plants and animals, Polycomb-group (Pc-G) genes mediate mitotically stable repression of targets such as homeotic genes that are critical for developmental patterning and growth control. An antagonistic group of proteins, the trithorax-group (trx-G), act as activators. Both groups act to maintain on/off patterns of transcription defined early in development, rather than to set up these patterns, and are important for maintaining cell fates. Although the Pc-G and trx-G have long been thought to cause epigenetic changes in chromatin structure, due to the stable but ultimately reversible alterations in gene activity they promote, the mechanistic basis for their activity has been mysterious. Recent studies have implicated histone modifications as an important component of epigenetic changes. A variety of modifications on the amino-tails of histones have been characterised, of which methylation of lysine residues has been thought to be particularly stable (Jenuwein and Allis, 2001; Fischle *et al*, 2003). The consequence of lysine methylation can differ both according to which lysine residue is modified and also as to how many methyl groups are added—lysine residues can be mono-, di- or trimethylated (Bannister *et al*, 2002). For example, methylation of histone H3 at lysine 9 (H3K9) or lysine 27 (H3K27) is generally correlated with transcriptional repression, whereas methylation at lysine 4 (H3K4) is predominantly associated with transcriptional activity (Jenuwein and Allis, 2001; Peters *et al*, 2003; Ringrose and Paro, 2004). In addition, the level of methylation is important, for example H3K9me3 shows a different distribution from H3K9me1 and H3K9me2 in mammals (Peters *et al*, 2003). Several enzymes with histone lysine demethylase activity have now been identified, indicating that methylation can be rapidly reversed (Shi *et al*, 2004; Tsukada *et al*, 2006).

In animals, Pc-G proteins have been biochemically purified in at least two distinct complexes, Polycomb repressive complex 1 and 2 (PRC1 and PRC2). In accordance with their epigenetic role, both complexes have been recently shown to modify histones. *Drosophila* PRC2 consists of the four core members SUPPRESSOR OF ZESTE 12 (SU(Z)12), P55, EXTRA SEX COMBS (ESC) and ENHANCER OF ZESTE (E(Z)) (Ringrose *et al*, 2004). E(Z) carries a histone methyltransferase domain, the conserved SET domain, and can trimethylate lysine 9 and 27 of histone H3 but requires other PRC2 members to accomplish its catalytic activity (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Muller *et al*, 2002). A detailed examination of histone methylation at Pc-G targets revealed that silencing of *Drosophila* Pc-G target genes is in most cases correlated with both H3K27me3 and H3K9me3, whereas presence of only one modification was not an indicator of silencing (Ringrose *et al*, 2004). Subsequently, the marks set by PRC2 may create a binding site for the chromodomain protein POLYCOMB (PC), which then recruits PRC1 (Cao *et al*, 2002; Czermin *et al*, 2002). Inhibition of chromatin remodelling by SWI/SNF complexes and direct compaction of nucleosomes by PRC1 might then lead to stable, long-term silencing (Francis *et al*, 2004). So far, no DNA-binding activity of core Pc-G proteins has been determined; thus, it is largely unresolved how they are recruited to their target genes. However, in *Drosophila*, many DNA elements, the so-called

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Polycomb repressive elements (PREs), that are both required and sufficient for recruitment of Pc-G complexes have been identified (Ringrose *et al*, 2003, 2004).

In plants, only the PRC2 is structurally conserved and most of its members are encoded by small gene families. Genetic and molecular analysis suggests that their products act in several PRC2-like complexes with discrete but overlapping functions (Hsieh *et al*, 2003; Reyes and Grossniklaus, 2003; Chanvivattana *et al*, 2004; Schubert *et al*, 2005). In *Arabidopsis*, there are three *E(z)* homologues: *MEDEA* (*MEA*), *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*, also known as *EZA1*). *MEA* predominantly acts during seed development, whereas *CLF* and *SWN* are expressed more generally in plants. *CLF* is required to repress floral homeotic genes such as *AGAMOUS* (*AG*) and also the homeobox gene *SHOOTMERISTEMLESS* (*STM*) (Goodrich *et al*, 1997; Katz *et al*, 2004). It is likely that *CLF* regulates many other targets, as there is substantial redundancy between *CLF* and the related *SWN* gene (Chanvivattana *et al*, 2004). There are also three *Su(z)12* homologues, *FERTILISATION INDEPENDENT SEED2* (*FIS2*), *VERNALISATION2* (*VRN2*) and *EMBRYONIC FLOWER2* (*EMF2*). The action of *FIS2*, like *MEA*, is largely confined to the seed, whereas *VRN2* and *EMF2*, like *CLF* and *SWN*, act more generally in development. *VRN2* is required for repression of *FLC*, an inhibitor of flowering, in response to cold treatments (vernalisation). *EMF2* has a similar role to *CLF* in repression of floral homeotic and other target genes. Double mutant analysis suggests that *EMF2* and *VRN2* also show redundancy, as double mutants have severe phenotypes and resemble *clf swn* doubles (Schubert *et al*, 2005). In contrast to the other *Arabidopsis* PRC2 homologues, *ESC* is represented by a single copy gene, *FERTILISATION INDEPENDENT ENDOSPERM* (*FIE*). Null *fie* mutants are embryonic lethal, like *fis2* and *mea* mutants; however, depletion of *FIE* activity later in development, for example by co-suppression, reveals that *FIE* likely acts with *CLF* and *EMF2* to repress common targets (Kinoshita *et al*, 2001; Katz *et al*, 2004). Thus, *clf swn* and *emf2 vrn2* double mutants, as well as plants lacking *FIE* activity, have similar phenotypes and likely lack vegetative Pc-G activity.

Several studies suggest that the plant PRC2 may also act as an H3K27 methyltransferase. Immunostaining experiments show that in wild-type plants H3K27me3 localises to euchromatin, whereas H3K27me2 strongly labels heterochromatin and has weaker staining in euchromatin. In *clf swn* mutants and in transgenic plants with severely reduced *FIE* activity, H3K27me2 staining was reduced at euchromatin but not at heterochromatin; H3K27me3 staining in euchromatin was also reduced but frequently became re-distributed to heterochromatin (Lindroth *et al*, 2004). These results suggested that the plant Pc-G control H3K27me2 and H3K27me3 in euchromatin, but that other genes can also supply this function particularly in heterochromatin. In addition, chromatin immunoprecipitation (ChIP) experiments have shown that silencing of *FLC* by *VRN2* is associated with H3K27me2 methylation (Bastow *et al*, 2004; Sung and Amasino, 2004). Despite this progress, the role of different histone methylation states in silencing of specific plant Pc-G targets remains poorly defined and for most targets it is not known whether the Pc-G act directly or indirectly. In addition, it is unclear how silencing of Pc-G target genes is inherited through

mitosis and whether Pc-G proteins are persistently required to maintain repression.

Here, we show that CLF is a nuclear-localised protein that is persistently required for silencing of *AG* in leaves but is unlikely itself to constitute a heritable epigenetic mark. Using ChIP, we show that plant Pc-G targets are characterised by dispersed H3K27me3 methylation that colocalises with CLF protein on chromatin. We discuss the possible functions of H3K27me3 spreading for the inheritance and stability of epigenetic silencing in plants.

Results

The SET domain is necessary for CLF+ activity

The strongest similarity between the CLF and E(Z) proteins lies in their SET domains, suggesting that like E(Z), CLF also acts as an HMTase. To confirm that the SET domain was required for *CLF+* activity, we characterised classical *clf* alleles to see if any had lesions within the SET domain. We found that the *clf-81* allele (Kim *et al*, 1998), which phenotypically resembles the null *clf-50* allele (Figure 1A), had a missense mutation that encoded the substitution R794H within the SET domain. Alignments indicated that the R794 residue is highly conserved between diverse SET domain proteins, including the human K4 H3 HMTase SET7/9 and the fission yeast K9 H3 HMTase CLR4 (Figure 1B). It lies in a helical region that is predicted from structural studies of the SET domain to be part of a groove that accommodates histone tails: for example, the corresponding residue (R258) of human SET7/9 is thought to bind the side chain of R2 on the histone H3 substrate (Xiao *et al*, 2003). The phenotype of the *clf-81* allele (Figure 1B) may therefore reflect impaired histone binding by the CLF HMTase. Consistent with this, we
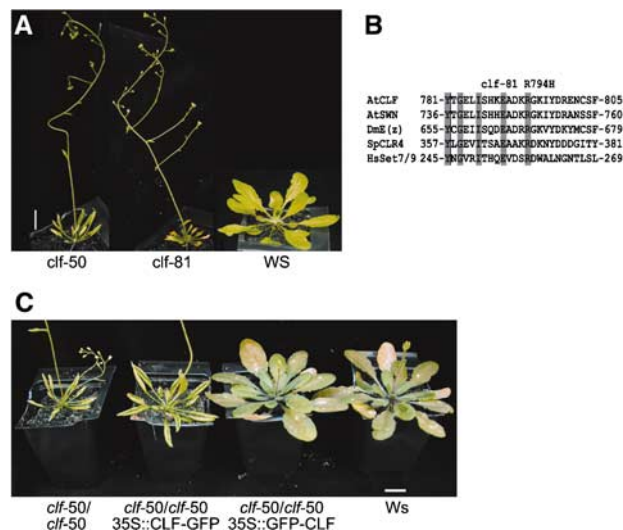

Figure 1 A severe *clf* allele carries a point mutation in the SET domain. (**A**) Alignment of a part of the SET domains of different SET domain proteins; residues conserved in all proteins are shaded, residue R794 that is mutated to H in *clf*-81 is marked. (**B**) *clf*-81 shows leaf curling and early flowering (compare to wild-type Ws (Wassilewskija)), similar to the *clf*-50 deletion allele. (**C**) *clf*-50 is complemented by a 35S::GFP-CLF transgene but only partially by a 35S::CLF-GFP transgene. All plants were grown under short day conditions; scale bar is 1 cm. AtCLF, *Arabidopsis thaliana* CURLY LEAF; AtSWN, *A. thaliana* SWINGER; DmE(z), *Drosophila melanogaster* ENHANCER OF ZESTE; SpCLR4, *Schizosaccharomyces pombe* CLR4; HsSET7/9, *Homo sapiens* SET7/9.

found that histone methylation was reduced in *clf-81* plants (see later results).

CLF protein is nuclear localised but is not present throughout mitosis

To localise the CLF protein, we made transgenes (*35S::GFP-CLF* and *35S::CLF-GFP*) that expressed CLF as a fusion with GFP, under control of the cauliflower mosaic virus 35S promoter. The *35S::GFP-CLF* construct fully complemented the null *clf-50* mutation in transgenic plants, whereas *35S::CLF-GFP* gave little or no complementation (Figure 1C). We did not observe any phenotypic abnormalities resulting from expressing *CLF* under the constitutive 35S promoter, perhaps because expression of the endogenous *CLF* gene is also fairly constitutive (Goodrich *et al*, 1997). Consistent with a role for CLF in chromatin modifications, microscopy indicated that the GFP-CLF fusion was predominantly localised to nuclei in transgenic plants (Figure 2). In contrast with the *Arabidopsis* Pc-G protein VRN1, which localises to metaphase chromosomes in root tips and is present throughout mitosis (Mylne *et al*, 2006), we did not observe any metaphase figures showing GFP-CLF fluorescence (compare Figure 2B and C). It is therefore unlikely that CLF itself constitutes a heritable epigenetic mark, as it is lost during mitosis.

The CLF protein is required persistently to silence AG

The *CLF* mRNA is expressed persistently during leaf and flower development (Goodrich *et al*, 1997). To test whether CLF protein is required persistently to maintain silencing, or can act in a 'hit and run' fashion, we made a transgenic line
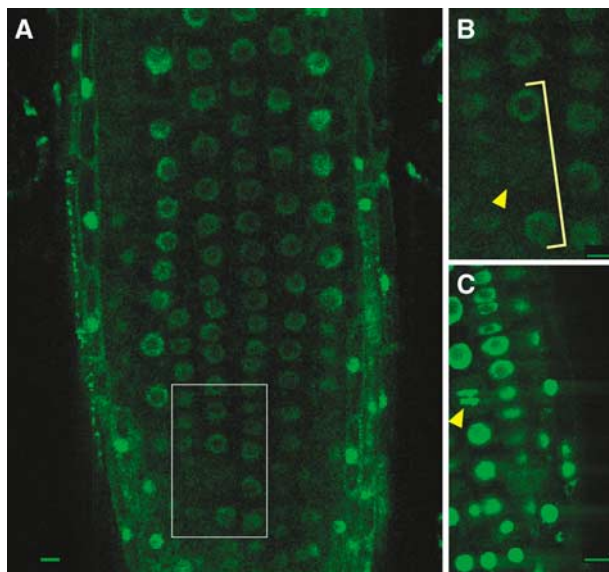

Figure 2 CLF protein is nuclear localised but is not present in nuclei throughout mitosis. (**A**) Root of a *clf-50/clf-50 35S::GFP-CLF* plant showing GFP expression in most cells in the nuclei. (**B**) Close-up of the inset in (A) showing a cell without nuclear GFP expression (arrowhead). Adjacent cells in the same cell file show nuclear GFP (bracket). (**C**) Details of a *VRN1::VRN1-GFP* root exhibiting a mitotic figure (arrowhead). At least 10 roots per line were analysed by confocal laser microscopy; in no case, GFP-stained mitotic figures were identified in *clf/clf 35S::GFP-CLF* roots, whereas *VRN1::VRN1-GFP* roots showed green-fluorescing mitotic figures in all roots. Scale bars, 10 μm.

(*clf-2 pCLF::CLF-GR*; see Materials and methods for details) in which *CLF+* activity is steroid dependent. Thus, plants that were supplied with dexamethasone (dex) steroid from germination onwards had a wild-type phenotype, whereas those grown in the absence of steroid had a *clf* mutant phenotype (Figure 3A–C). To monitor *AG* activity, we further introduced the *pAG-I::GUS* reporter transgene, which carries the *cis*-acting sequences necessary for response to *CLF* (Sieburth and Meyerowitz, 1997), into this conditional *CLF* background. To test the requirement for *CLF* to maintain silencing of *AG*, we grew plants on agar plates containing steroid for 10 days, then transferred the seedlings to soil and either withdrew (−dex) or maintained (+dex) steroid supply. At the time of the shift, plants had 4–5 visible leaves (>3 mm in size) (Figure 3D) and microscopy indicated that all rosette and cauline leaves had been initiated and flowers were initiating at the shoot apex (Figure 3E). The morphology of the shifted plants was scored 17 days after the shift when they had bolted, and rosette leaves were fully expanded. Unlike the plants grown continuously on steroid, those that were removed from steroid developed curled rosette leaves, particularly in leaves 5 and 6 (Figure 3C). Staining the plants for *pAG-I::GUS* activity confirmed that the rosette leaves 5 and 6 had strong staining compared to plants grown on steroid throughout (Figure 3F and G). This suggested that expression of *CLF* activity transiently during early leaf development is not sufficient to maintain silencing of *AG* in leaves.

AG and STM are direct targets of CLF

AG appears to be a principal target of *CLF*, as the *clf* mutant phenotype is largely dependent on *AG+* activity (Goodrich *et al*, 1997). However, it was not known whether CLF acted directly on *AG* or indirectly, for example by repressing an activator of *AG*. To address this, we used ChIP to analyse the sequences bound by CLF *in vivo*. Because we lacked an antibody specific for CLF, we used an epitope-tag strategy: chromatin from *35S::GFP-CLF clf-50* seedlings was immunoprecipitated (IP) using an α-GFP antibody that is reliable for ChIP (Vanoosthuyse *et al*, 2004). We found strong enrichment for sequences from multiple sites within the *AG* locus (Figure 4C and data not shown). However, we did not detect enrichment for the genes upstream or downstream of *AG* (Figure 4D) or for heterochromatic sequences such as TA3 (Figure 4B). In addition, we did not see enrichment for *AG* sequences in similar ChIP experiments using a control *35S::GFP* transgenic line (Supplementary Figure S3). Together, these results suggested that CLF binds *AG in vivo*, and is dispersed over multiple sites on *AG* chromatin.

We next asked whether the homeobox gene *SHOOT MERISTEMLESS* (*STM*), which is mis-expressed in *clf* mutants (Katz *et al*, 2004), is also bound by CLF. Indeed, further ChIP analyses revealed binding of CLF to *STM*. The binding of CLF at *STM* was weak relative to *AG*, but again appeared to be dispersed over the locus (Figure 4E) but not the neighbouring genes (Figure 4F).

The AG and STM loci are covered with the repressive mark H3K27me3 and lack the active mark H3K4me2

We next tested whether *AG* and *STM* carry the repressive histone methylation marks associated with Pc-G repression (H3K27me2, H3K27me3) using chromatin from whole seed-
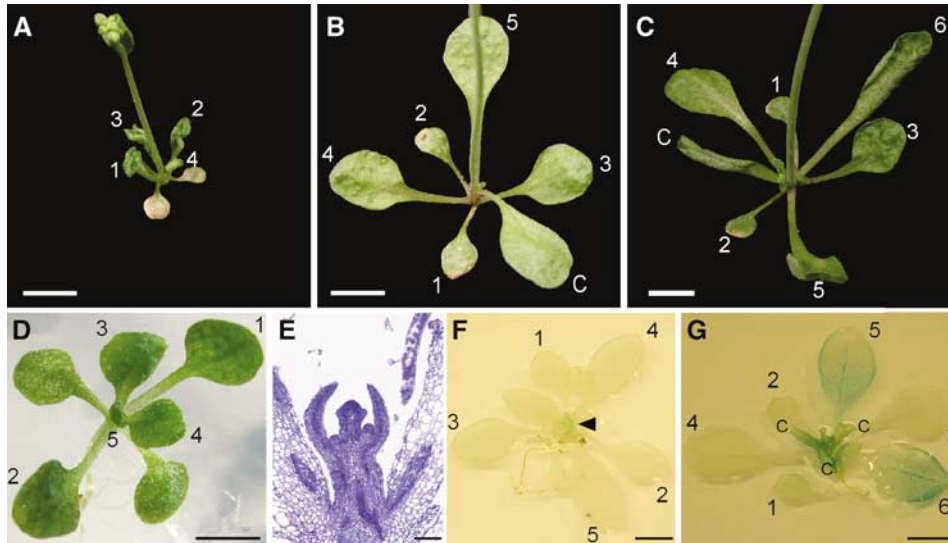
Figure 3 *CLF* is persistently required for repression of *AG* in leaves. (A–G) All plants are *clf/clf* and carry the *pCLF∷CLF-GR* and the *pAG-I∷GUS* transgenes. Plants were initially grown on MS medium under long-day condition with or without dex steroid and transferred at day 10 to soil and either sprayed or not sprayed with dex. (**A**) A 27-day-old plant grown without dex throughout. (**B**) A 27-day-old plant grown with dex throughout. (**C**) A 27-day-old plant grown for 10 days with dex, then transferred from soil and dex withdrawn. Note leaf curling of leaves 4 and 5 and cauline leaf (C). (**D**) Plant grown on dex for 10 days shows no leaf curling. (**E**) Longitudinal section through a 10-day-old seedling grown with dex. Note that all leaf primordia have formed and that the plant has started flowering, so that the influorescence bolt is visible. (F, G) GUS staining reflects *AG* expression. (**F**) A 27-day-old plant grown on dex throughout only shows GUS staining in the flowers (arrowhead). (**G**) A 27-day old plant shifted on day 10 from dex to no dex shows strong GUS staining in leaves 5 and 6 and cauline leaves. Numbers indicate leaf number emerging after germination. Scale bars: 5 mm (A–C, F, G), 2 mm (D), 50 μm (E).

lings, that is, tissues in which both genes are repressed in a Pc-G-dependent fashion. To confirm the specificity of the antibodies for different histone marks, we first tested heterochromatic sequences from the TA3 retrotransposon in ChIP assays. Consistent with previous reports (Lindroth *et al*, 2004; Mathieu *et al*, 2005; Naumann *et al*, 2005), we detected strong enrichment for H3K27me2 but not for H3K4me2 and H3K27me3 (Figure 5C). Next, we examined the CLF target genes: H3K27me3 covers the *AG* and *STM* loci but does not extend to the flanking genes, similar to the distribution observed for CLF (Figure 5A–D). Although microarray studies (see Genevestigator in Zimmermann *et al*, 2004) suggest that like *AG* and *STM*, the gene upstream of *STM* (At1g62370) is not detectably expressed in leaves, it notably lacked H3K27me3; thus, this is not a generic mark for transcriptionally inactive genes. Additionally, we detected weak, but reproducible enrichment of H3K27me2 in regions B, F and G of *AG* and regions 1 and 3 of *STM* (Figure 5A and C). B lies in the *AG* promoter region, whereas F is in a region of the *AG* second intron, which is highly conserved in different plant species (Hong *et al*, 2003). Thus, in contrast to H3K27me3, H3K27me2 appears to localise more specifically. Silencing of *AG* and *STM* was also reflected in reduced enrichment compared to *ACT* or *PFK* for H3K4me2, a mark that is predominantly found on active genes (Gendrel *et al*, 2002) (Figure 5A and C).

The above ChIP experiments were performed on 12-day-old seedlings, in which most of the tissue is from young leaves. To test whether the methylation profiles changed during leaf development, we performed ChIP using fully expanded leaves from plants that were flowering. The results for H3K27me2 and H3K27me3 at *AG* and *STM* were similar to those for seedlings (Supplementary Figure S2). This sug-

gested that the repressive H3K27 methylation marks persist throughout leaf development.

H3K27me3 is dependent on Pc-G activity

As CLF protein and H3K27me3 colocalise on *AG*, we asked whether this methylation is dependent on *CLF*+ activity. Indeed, coverage of *AG* with H3K27me3 was almost completely lost in *clf* mutants, whereas H3K27me2 was only slightly affected (Figure 5A). In *emf2-10* mutants, which have a weak emf2 phenotype resembling that of *clf* mutants, *AG* is also derepressed (Chanvivattana *et al*, 2004). Consistent with the proposed role of EMF2 and CLF in a common complex, *emf2-10* mutants had very similar effects on H3K27 methylation to *clf* mutants (Figure 5A). In addition, regions E and F of *AG* gained H3K4me2 in the mutants, consistent with activation of *AG* (Figure 5). Importantly, H3K27me3 at the *AG* locus was also lost in *clf-81*, confirming the importance of the SET domain for CLF function and histone methylation (Supplementary Figure S1).

Unlike *AG*, there was little change in histone methylation at *STM* in *clf* or *emf2-10* single mutants (Figure 5C). However, previous genetic studies suggested that *CLF* has partial redundancy with the related gene *SWN*, and similarly *EMF2* does so with *VRN2* (Chanvivattana *et al*, 2004; Lindroth *et al*, 2004; Schubert *et al*, 2005). Consistent with this, H3K27me3 was completely lost in all regions tested in both *clf swn* and *emf2 vrn2* double mutants and H3K4me2 was correspondingly gained in regions 2–5 (Figure 5C). In addition, expression of STM is stronger in *clf swn* double mutants than in *clf* single mutants (Figure 5E). Thus, strong *STM* mis-expression in the double mutants is strictly correlated with loss of H3K27me3 and gain of H3K4me2, whereas weak mis-expres-
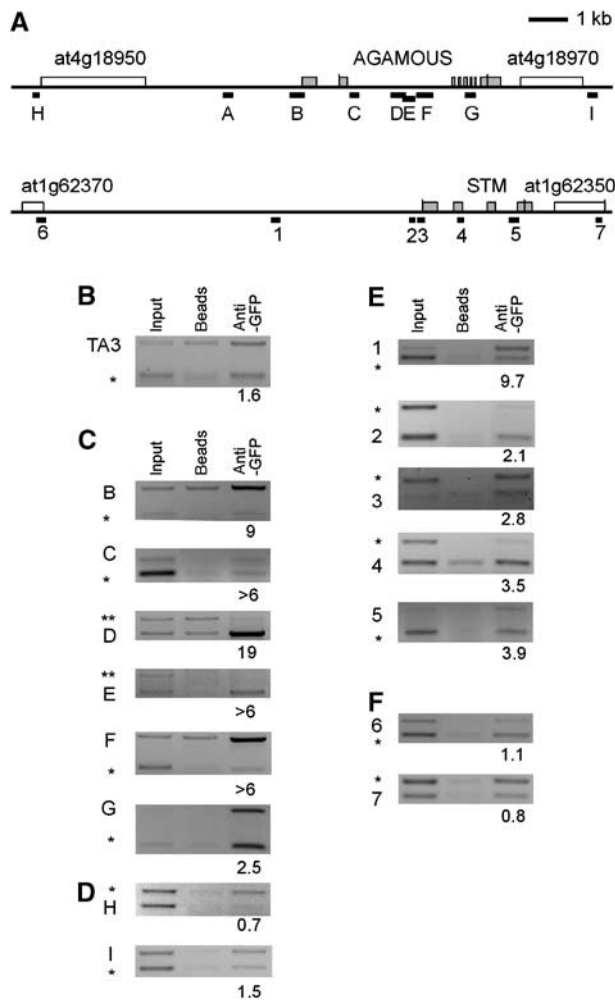
Figure 4 CLF is bound to *AG* and *STM*. (**A**) Schematic structure of the *AG* and *STM* loci including the 5′- and 3′-flanking genes. For *AG* and *STM*, the exon/intron structure (exons are shaded), and for the flanking genes, the transcribed regions are depicted. Black bars with letters (for *AG*) or numbers (for *STM*) indicate regions amplified in ChIP PCRs. (B, C) IP on chromatin preparations isolated from 10-day-old seedlings of a *35S::GFP-CLF* transgenic line was performed without antibody ('beads') or anti-GFP antibodies and precipitated DNA was amplified by PCR. Semiquantitative ChIP duplex PCRs were used to amplify *ACTIN 2/7* (marked with *) or *PHOSPHOFRUCTOKINASE* (PFK) sequences (marked with **) as internal controls and regions from *TA3* retrotransposon (**B**) as negative control, from *AG* (**C**) and *AG* flanking genes (**D**), or from *STM* (**E**) and *STM* flanking genes (**F**). Numbers below the lanes indicate the ratio of the intensity of *TA3*, *AG* or *STM* products, respectively, compared to *ACTIN* or *PFK* intensity after IP normalised to the ratio before IP ('input'). Each experiment was performed at least twice; representative experiment is shown.

sion in the single mutants is accompanied by only slight changes in histone methylation at the *STM* locus.

Vernalisation induces VRN2-dependent gain of H3K27me3 on FLC but not its neighbours

Two previous studies have revealed that the *FLC* locus acquires VRN2-dependent H3K27me2 upon vernalisation (Bastow *et al*, 2004; Sung and Amasino, 2004). In addition, the *FLC* neighbouring genes at5g10130 and at5g10150 are downregulated during prolonged cold treatment, suggesting that silencing initiated at *FLC* might spread upstream and downstream (Finnegan *et al*, 2004). As we detected coverage

of *AG* and *STM* with H3K27me3, we wondered whether coordinated downregulation of *FLC* and its neighbouring genes might reflect spread of H3K27me3 beyond FLC. Thus, we used ChIP to study changes in histone methylation before and after vernalisation (40 days at 4°C, then 15 days at 22°C under long-day conditions) in *fca* mutants (a vernalisation requiring background) and compared this to backgrounds in which vernalisation is impaired (*vrn1 fca, vrn2 fca*). Indeed, the *FLC* locus acquired significantly higher levels of H3K27me3 after vernalisation in regions 2, 3 and 4 (4- to 12-fold); thus, is likely covered with H3K27me3, similar to the pattern at *AG* and *STM* (Figure 6 and Supplementary Figure S4). Importantly, the gain of H3K27me3 was dependent on *VRN2* but not *VRN1*: unlike *vrn2*, *vrn1* mutations reduced but did not eliminate the rise in H3K27me3 after vernalisation (Figure 6 and Supplementary Figure S4). Although H3K27me3 covered *FLC*, it did not spread to the neighbouring genes: *At5g10150* was devoid of H3K27me3 and *At5g10130* showed only slight enrichment of H3K27me3 after vernalisation. We also observed enrichment of H3K27me2 after vernalisation at *FLC* in *fca*, but to a lower extent than H3K27me3 (two-fold; Figure 3 and Supplementary Figure S4).

H3K27me3 spreads on an AG transgene in a CLF-dependent manner

Because H3K27me3 and CLF cover large parts of *AG*, it is unlikely that they are recruited to all regions of *AG* in a DNA-sequence-specific manner. Thus, it is possible that initial recruitment of CLF and therefore H3K27me3 is guided by DNA-sequence specific factors and then spreads over the gene to achieve stable silencing. To reveal whether H3K27me3 can spread from *AG* sequences to neighbouring sequences, we performed ChIP on transgenic plants that carry a transgene (*pAG-I::GUS*) that carries the *cis*-acting *AG* sequences necessary for regulation by CLF (Sieburth and Meyerowitz, 1997). The construct carries the 3′ portion of the gene (*At4g18950*) upstream of *AG*, the intergenic region between *At4g18950* and *AG*, and the 5′-*AG* sequences extending to the third exon (Figure 7A). We amplified sequences that are only present on the transgene (β-GLUCURONIDASE (*GUS*) and NEOMYCIN PHOSPOTRANSFERASE (*NPT*)) and therefore are in close proximity to the AG regulatory regions (promoter and large intron) (Figure 7A). We detected strong enrichment for H3K27me3 on *AG* and *GUS* sequences in wild type but not in *clf* mutants carrying the transgene (Figure 7B). Because the *GUS* gene is not an endogenous Pc-G target, this suggests that H3K27me3 is initiated at *AG* sequences and spreads onto neighbouring sequences in a *CLF*-dependent manner. By contrast, H3K27me3 was only slightly enriched at the *NPT* gene, similar to *At4g18950* in the native *AG* context, suggesting that it did not spread upstream of the *AG* gene. In addition, strong enrichment for H3K4me2 was observed in wild type at the *NPT* gene, but only little at *GUS*, consistent with the *NPT* gene being expressed and the *GUS* gene being silenced. In *clf* mutants, where *pAG-I::GUS* is de-repressed, the *GUS* gene showed much stronger H3K4me2 enrichment.

H3K27me3 is not sufficient for silencing

The *pAG::GUS* transgene lacks *AG* intragenic regulatory sequences and is expressed in leaves (Sieburth and
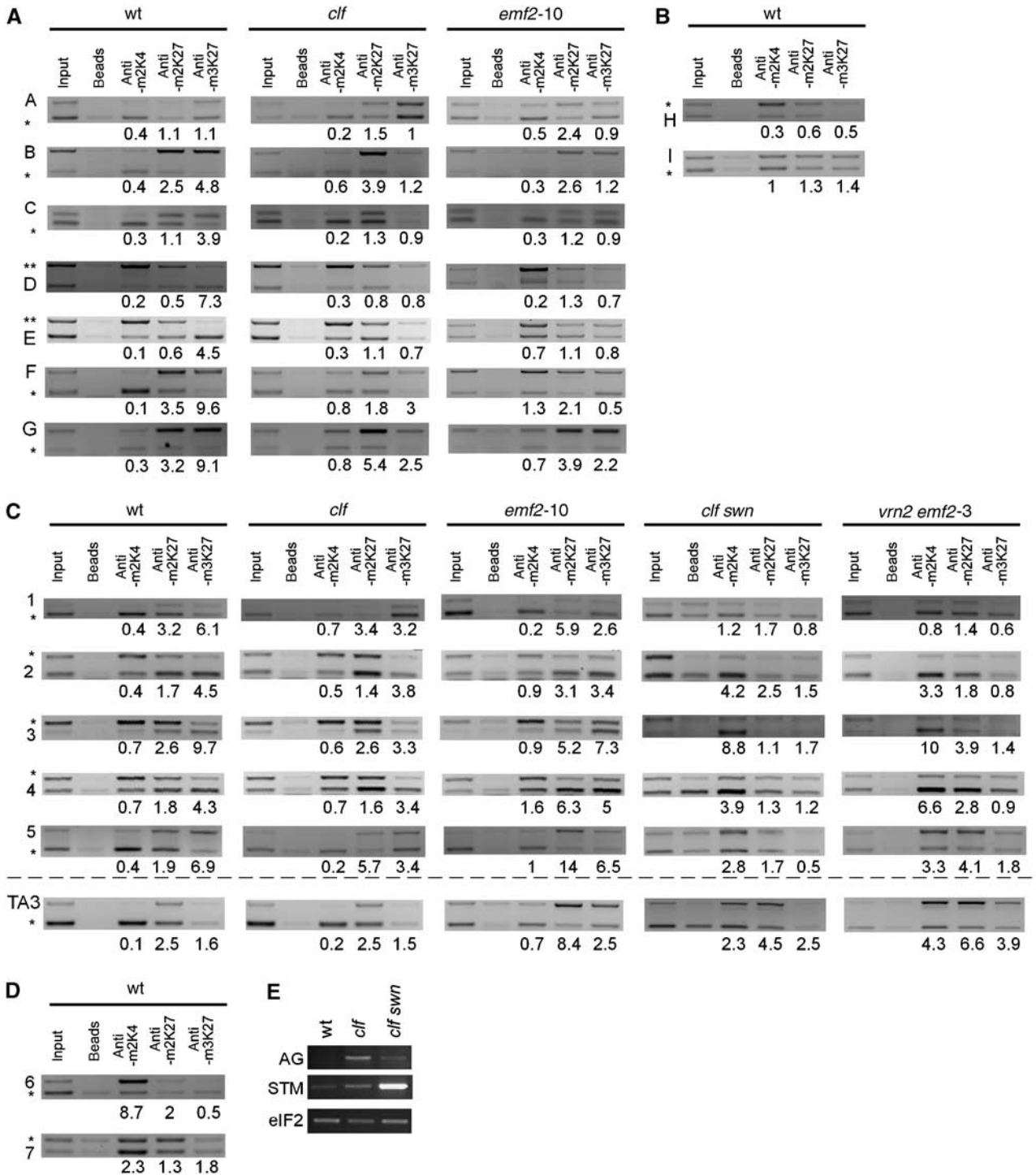
Figure 5 Histone methylation patterns of *AG* and *STM* in wild-type and Pc-G mutant plants. (A–D) ChIPs were performed with antibodies against H3K4me2, H3K27me2 and H3K27me3 from wild-type (A–D), *clf* (A, C), *emf2* (A, C), *clf swn* (C) and *vrn2 emf2-3* (C) chromatin extracted from 10-day-old seedlings. Regions amplified are shown in Figure 4A and calculation of relative enrichment is described in Figure 4. Results of ChIP PCRs on the *AG* locus (**A**), on the *AG* flanking genes (**B**), the *STM* locus and *TA3* retrotransposon (latter is H3K27me2control) (**C**) and on the *STM* flanking genes (**D**). (**E**) RT–PCRs with primers amplifying *AG*, *STM* or *eIF2* coding regions on cDNAs generated from mRNA isolated from wild-type, *clf* and *clf swn* seedlings. *EIF2* was used as a loading control.

Meyerowitz, 1997). Consistent with this, H3K4me2 enrichment at the *GUS* gene was greater than in the silenced *pAG-I*::*GUS* (Figure 7B). Unexpectedly, we also found strong enrichment for H3K27me3 at *GUS* sequences. Thus, H3K27me3 is necessary for silencing of Pc-G targets but is not sufficient.

Discussion

Previous studies have suggested that CLF acts in a complex similar to PRC2 and that it likely regulates histone methylation. In particular, immunostaining experiments have shown global effects of the plant Pc-G genes on H3K27 methylation
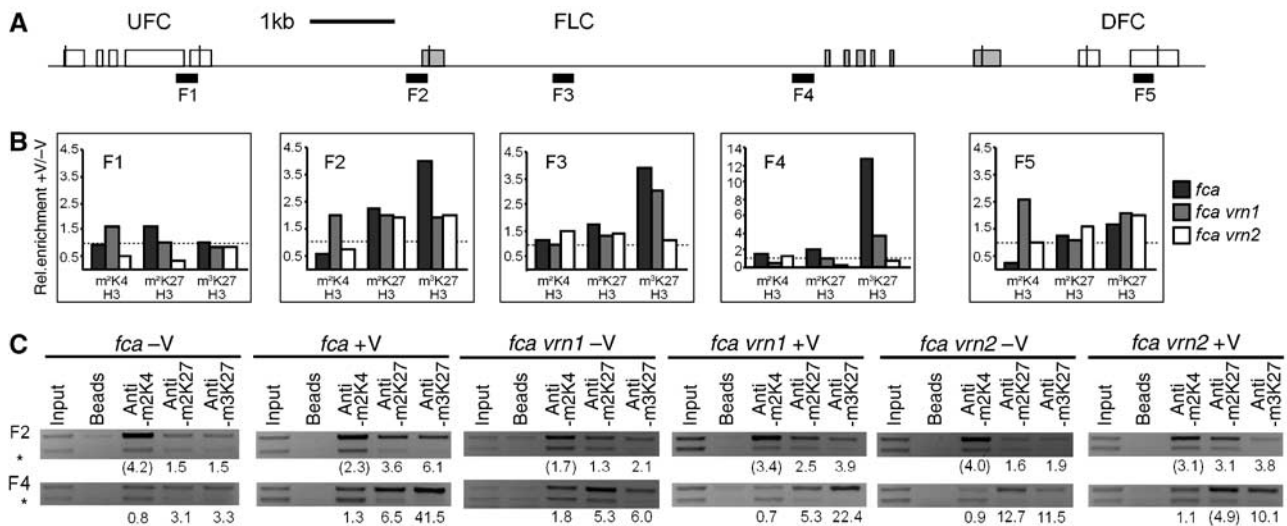
Figure 6 The entire *FLC* locus shows strong enrichment with H3K27me3 after vernalisation. (**A**) Schematic structure of the *FLC* locus including the flanking genes *UFC* and *DFC*. The *FLC* exons are shaded. Black bars indicate the regions amplified. (**B**) Summary of ChIP PCRs performed with antibodies against H3K4me2, H3K27me2 and H3K27me3 on chromatin extracted from the vernalisation-requiring background *fca*, *vrn1 fca* and *vrn2 fca* mutants before and after vernalisation. Plants were grown for either 2 days at 4°C (−V) or 40 days at 4°C (+V), then transferred to 22°C and harvested after 15 days. Relative enrichment for ChIPs was determined as described in Figure 4 and the ratio of +V/−V for each genotype and antibody was calculated. Dashed lines indicate relative enrichment of 1. (**C**) Example of ChIPs performed for the different genotypes, antibodies and growth conditions. The whole data set is available in Supplementary Figure S4.

in euchromatin and heterochromatin. Here, we use ChIP to dissect how CLF and SWN control methylation patterns at specific targets. We show that CLF likely acts in several different complexes with different HMTase specificities. As with the animal PRC2, silencing by Pc-G requires H3K27me3; however, in plants, the H3K27me3 is spread throughout the chromatin of their targets. This may be significant for regulation of clusters of genes and for the disruption of silencing that occurs when transposons insert at Pc-G targets.

CLF and SWN mediate dispersed H3K27me3 methylation at their targets

Although *CLF* shows considerable redundancy with the related *SWN* gene, there is an absolute requirement for *CLF+* activity for silencing of *AG* during vegetative development; thus, the effects of Pc-G on chromatin were most easily revealed for *AG*. We found a strong enrichment for H3K27me3 that extended over all regions of the *AG* locus that we tested, including the promoter, intron and exon regions. Importantly, this methylation was strictly dependent on *CLF+* activity, as it was lost in *clf* mutants. It was also lost in *emf2-10* mutants, consistent with the EMF2 and CLF proteins acting together in a common complex with HMTase activity (Chanvivattana *et al*, 2004). We also observed strong enrichment for H3K27me3 at two other Pc-G target genes, *STM* and *FLC*. Again, the methylation was not localised to specific regions of the targets but appeared to be dispersed throughout the promoter and transcribed regions. This contrasts with the punctate pattern of H3K27me3 that has been described in *Drosophila*, where it has been localised to discrete regulatory elements, the PREs (Cao *et al*, 2002; Ringrose *et al*, 2004; Wang *et al*, 2004). However, several recent animal studies have also revealed dispersed H3K27me3 methylation (Bernstein *et al*, 2006; Lee *et al*, 2006; Tolhuis *et al*, 2006).

The *clf* mutant phenotype is enhanced by *swn* mutations, suggesting that the two genes act redundantly (Chanvivattana *et al*, 2004). Presumably, there are some targets at which either *CLF* or *SWN* activity is sufficient for silencing so that they are only significantly mis-expressed in *clf swn* double mutants. Consistent with this, *STM* expression was much higher in *clf swn* double mutants that in the single mutants. This correlated well with the effects of the mutants on histone methylation at *STM*. The H3K27me3 enrichment at *STM* was depleted in *clf swn* double mutants, whereas the single mutants had similar levels to wild type. We observed similar redundancy between *EMF2* and *VRN2* for H3K27me3 enrichment at *STM*. We conclude that CLF/SWN and EMF2/VRN2 can act for the most part interchangeably in PRC2-like complexes with H3K27me3 HMTase activity. The catalytic activity of these complexes is likely provided by the SET domains of CLF and SWN. This is supported by our characterisation of the *clf-81* allele, which demonstrates that a conserved residue in the SET domain that has been implicated in binding histones is necessary for H3K27me3 methylation at *AG*.

We were also able to directly localise CLF to *AG* and *STM* chromatin by ChIP analysis of transgenic lines that expressed epitope-tagged CLF (35S::GFP-CLF) and observed a dispersed distribution. An obvious concern is that the distribution of GFP-CLF does not reflect that of the endogenous CLF protein, for example the 35S promoter might confer abnormally high levels of CLF protein that lead to spurious associations. We think this unlikely for two reasons. Firstly, the *35S::GFP-CLF* transgene fully complements the null *clf-50* mutation but does not cause any gain-of-function phenotypes that might reflect ectopic *CLF+* activity. Secondly, the distribution of the GFP-CLF fusion protein in our transgenic lines precisely matches that of H3K27me3 in wild-type (non-transgenic) backgrounds. Importantly, it does not spread any further from the *AG* locus than does H3K27me3 in wild type.
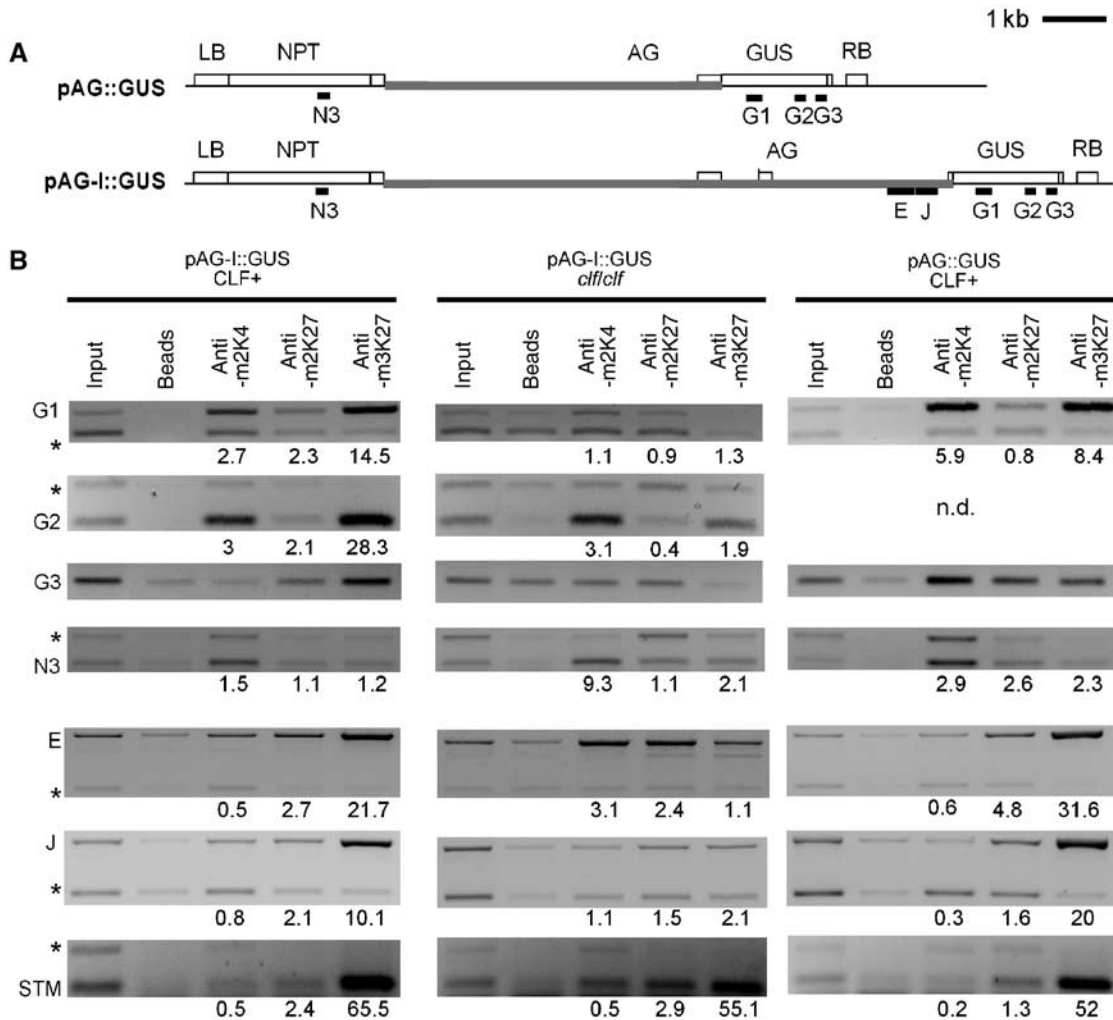
Figure 7 H3K27me3 spreads over *AG* transgenic sequences but is not sufficient for silencing. (**A**) Schematic structure of the *pAG::GUS* and *pAG-I::GUS* transgenes, constructed by Sieburth and Meyerowitz (1997). Black bars indicate regions amplified in ChIP PCRs. Grey bars indicate sequences homologous to *AG*. The *pAG::GUS* T-DNA contains a *NEOMYCIN PHOSPHOTRANSFERASE* (*NPT*) resistance gene close to the left border (LB) of the T-DNA, around 6 kb of *AG* upstream region, the first *AG* exon, the β-*GLUCURONIDASE* (*GUS*) coding region and the right border (RB) of the T-DNA. The *pAG-I::GUS* T-DNA consists of the same T-DNA backbone and *AG* upstream region but contains the first two exons and the first two introns of *AG*. The *pAG-I::GUS* reporter reflects the endogenous *AG* expression pattern in wild-type and *clf* leaves, whereas *pAG::GUS* is strongly mis-expressed in wild-type cotyledons and leaves (Sieburth and Meyerowitz, 1997). (**B**) Results of ChIP PCRs performed on IP with antibodies against H3K4me2, H3K27me2 and H3K27me3 on chromatin samples extracted from 10-day-old wild-type or *clf* seedlings carrying the *pAG-I::GUS* transgene or wild-type seedlings carrying the *pAG::GUS* transgene. Regions F and J of *AG* are present both at the endogenous *AG* locus and on the *pAG-I::GUS* transgene. A region of *STM* (region 2 in Figure 4) served as an H3K27me3 control. Relative enrichment for ChIPs was determined as described in Figure 4.

We conclude that the distribution of GFP-CLF faithfully mimics that of the endogenous CLF protein. Therefore, *AG* and *STM* are direct targets of CLF and so CLF must act as a transcriptional repressor, at least for these two targets. The enrichment for GFP-CLF at *STM* was less than at *AG*, consistent with the redundancy between SWN and CLF for silencing of *STM*.

Pc-G targets show localised H3K27me2 enrichment

Immunostaining experiments have shown that H3K27me2 is chiefly found in heterochromatic regions, with a low level enrichment for euchromatin, whereas H3K27me3 is absent from heterochromatin (Lindroth *et al*, 2004; Mathieu *et al*, 2005; Naumann *et al*, 2005). Consistent with this, our ChIP experiments showed that TA3, a retrotransposon confined to heterochromatin, was enriched for H3k27me2 but not

H3K27me3. In addition, we found enrichment for H3K27me2 at discrete regions of Pc-G targets, again suggesting that this mark is not exclusively heterochromatic. The levels of enrichment were modest relative to H3K27me2 at TA3 or H3K27me3 at *AG*. However, the results were very reproducible, for example we found H3K27me2 in region F of *AG* in more than 10 independent ChIP experiments. Unlike our results for H3K27me3, H3K27me2 methylation was not consistently eliminated in either *clf swn* or *emf2 vrn2* double mutant backgrounds although it was reduced. This agrees with the immunostaining results, which showed that heterochromatic regions retain H3K27me2 in severe Pc-G mutants, whereas the staining in euchromatin is reduced (Lindroth *et al*, 2004). It is possible that the residual H3K27me2 methylation reflects further redundancy, for example between *MEA* and *CLF/SWN*. This is supported by the observa-
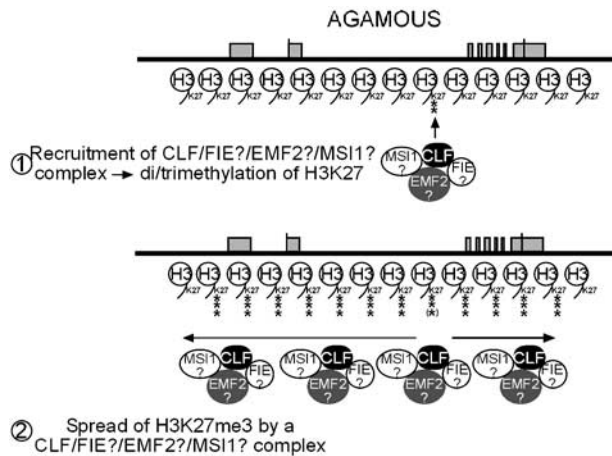
Figure 8 Model of the regulation of *AG* (and other Pc-G targets) by plant Pc-G proteins and H3K27 methylation. EMF2 and MSI1 are likely part of at least one of the putative complexes, as a reduction in their protein level causes phenotypes similar to *clf* and lines with reduced *FIE* levels.

tion that *MEA* is mis-expressed in leaves in *clf* mutants (Katz *et al*, 2004; Jullien *et al*, 2006). However, in plants lacking *FIE* activity, H3K27me2 is also retained at heterochromatin (Lindroth *et al*, 2004). Because *FIE* is single copy, and therefore unlikely to be redundant, this suggests that proteins other than Pc-G can provide H3K27me2 HMTase activity in plants. In addition to *CLF/SWN/MEA*, the *Arabidopsis* genome encodes at least a further 26 SET domain proteins that could potentially contribute to this activity (Baumbusch *et al*, 2001).

The coverage with H3K27me3 at the CLF target loci argues against a DNA-sequence-specific recruitment of CLF to all modified histones. The DNA region in the *AG* intron that shows H3K27me2 is highly conserved between different plant species and carries sequences required for both activation and repression of *AG* (Busch *et al*, 1999; Deyholos and Sieburth, 2000; Hong *et al*, 2003). One possibility is that this corresponds to a plant PRE and is responsible for initial recruitment of a CLF-containing complex, after which H3K27me3 methylation spreads over the target (Figure 8). The specificity of CLF as a di- or trimethylase may vary according to its partners—for example, human EZH2 occurs in several complexes with subtly different composition and these have different HMTase specificities (Kuzmichev *et al*, 2004). It will be interesting to test whether this region is sufficient to confer Pc-G-mediated silencing in novel contexts, as this is a defining feature of animal PREs.

H3K27me3 can spread beyond AG sequences but is not sufficient for silencing

If the dispersed methylation observed at *CLF* targets represents spreading from a localised initiation point, then methylation should spread beyond target sequences when they are juxtaposed in a novel context. To test this, we analysed histone methylation at the *pAG-I*::*GUS* transgene, previously shown to contain all the *cis*-acting sequences required for regulation by *CLF* (Sieburth and Meyerowitz, 1997). Consistent with this, we found that H3K27me3 was recruited to the transgene in wild-type but not *clf* mutant leaves. Importantly, sequences exclusively present on the transgene (*GUS*) were enriched for H3K27me3, suggesting that

H3K27me3 spreads from the *AG* intron to the 3′ transgenic sequences. The transgene also contained an *NPT* gene about 6 kb 5′ to the *AG* transcription start and this lacked H3K27me3. The gene *At4g18950* is located in a similar position 5′ to the endogenous *AG* locus, and also lacks H3K27me3. Thus, there may be sequences 5′ of *AG* that prevent spreading into neighbouring genes, similar to insulators described in other systems (Scott *et al*, 2006). Alternatively, because transgenic plants were selected on the basis of *NPT* gene expression, this could have selected for lines in which methylation had not spread into the *NPT* gene. In general, none of the *STM* and *AG* 5′ or 3′ flanking genes carried H3K27me3, suggesting either that spreading is short range or that these target genes are flanked by insulators.

The *pAG*::*GUS* transgene lacks important regulatory elements present in the large second *AG* intron and is de-repressed in leaves (Sieburth and Meyerowitz, 1997). Unexpectedly, we found that this transgene also recruits H3K27me3, which spreads into the *GUS* sequences. This suggests that H3K27me3 is necessary but not sufficient for Pc-G-mediated silencing. This is also supported by our analysis of the *FLC* gene. In addition to the changes in H3K27me2 described previously, vernalisation treatments resulted in increased H3K27me3 methylation at *FLC*. In *vrn1* mutants, in which silencing of *FLC* after vernalisation is impaired, H3K27me3 was only slightly reduced.

Functional significance of H3K27me3 spreading

Although spreading of silencing from heterochromatic sequences into neighbouring sequences is well established in flies and yeast, there is less evidence for this occurring in plants, so it is not clear if the spread of H3K27me3 at Pc-G targets could mediate spreading of silencing to neighbouring sequences. It has been reported that vernalisation treatments cause the genes flanking *FLC* to be downregulated as well (Finnegan *et al*, 2004). However, the flanking genes did not acquire H3K27me3, which may account for the fact that, unlike *FLC*, their silencing is not stably maintained after vernalisation (Finnegan *et al*, 2005). Thus, vernalisation may induce a widespread remodelling of chromatin at the *FLC* locus including histone deacetylation (Finnegan *et al*, 2005), but maintenance of silencing and H3K27me3 is then restricted to *FLC*. Although for *FLC*, *AG* and *STM*, H3K27me3 was locus specific, other Pc-G targets might occur in clusters with dispersed H3K27me3. The *Arabidopsis* genome contains many islands of coexpressed, neighbouring genes, which might correspond to regions with a common chromatin structure and epigenetic mark such as H3K27me3 (Ma *et al*, 2005; Ren *et al*, 2005; Schmid *et al*, 2005; Zhan *et al*, 2006).

Within target loci, dispersed H3K27me3 is necessary for stable silencing. Thus, *clf* mutants retain localised H3K27me2 at *AG*, but lack dispersed H3K27me3 and *AG* silencing. Dispersed H3K27me3 chromatin may help propagation of chromatin modifications through mitosis: if parental nucleosomes are randomly distributed between daughter chromatids during mitosis, there would be a low fidelity of inheritance if only a few nucleosomes carry the modification (Henikoff *et al*, 2004). However, if many nucleosomes have the modification, fidelity and epigenetic stability would be ensured. It is also important to note that the PRC1, which is thought to confer stable, long-term silencing, is not structu-

rally conserved in plants (Goodrich and Tweedie, 2002). Thus, coverage with H3K27me3, CLF and possibly other Pc-G proteins could manifest stable silencing through development independent of PRC1.

It is also notable that insertion of transposable elements at plant Pc-G target genes is often associated with a loss of silencing. This has been described for numerous gain-of-function alleles of the *Antirrhinum AG* and *STM* homologues *PLENA* and *HIRZINA*, and for the maize *STM* homologue *KNOTTED* (Bradley *et al*, 1993; Greene *et al*, 1994; Golz *et al*, 2002). A puzzling feature is that insertions at many different sites within the gene can all prevent silencing, so that it is unlikely that the failure simply results from disruption of a target site for a repressor. One possible explanation is that insertions prevent spreading of the H3K27me3 methylation at the target, and so disrupt silencing. Plant transposons are associated with distinct epigenetic marks, such as DNA methylation and H3K9me2 methylation (when silenced) or H3Kme2 (when active), that could inhibit spread of H3K27me3 methylation. It is striking that a recent study in mice revealed that large domains of H3K27me3 enrichment are significantly depleted for transposon sequences (Bernstein *et al*, 2006).

CLF is required persistently for silencing

Although epigenetic changes are by definition self-perpetuating, their propagation through cell division usually requires maintenance machinery—for example, the DNA methyltransferase *MET1* is needed for maintenance of mCpG methylation in plants (reviewed by Chan *et al*, 2005). H3K27 histone methylation persists throughout leaf development, as it was present at *AG* in mature leaves as well as in young seedlings. In addition, H3K27me3 is necessary for silencing at Pc-G targets, as in all cases where it was lost, silencing was also lost. Using a line with steroid-dependent *CLF* activity, we found that loss of CLF activity resulted in leaf curling and ectopic *AG* expression in leaves that were recently emerged at the time that plants were withdrawn from steroid media. Older leaves were less affected, perhaps because cell division had finished by the time that steroid had declined below a critical level in the shifted plants. The results suggest that CLF is required persistently to keep *AG* silenced in leaves, presumably to restore histone methylation after DNA replication, when it is thought that parental histones and newly synthesised histones are randomly assorted between the two daughter DNA chains (Henikoff *et al*, 2004; Annunziato, 2005). It is unlikely that CLF itself provides an epigenetic tag, as it is lost from chromosomes during mitosis. Thus, histone methylation itself or other Pc-G proteins may confer mitotically heritable silencing.

In summary, we show that dispersed H3K27me3 is a signature of plant Pc-G target genes. Use of whole genome approaches, such as ChIP on chip, will help clarify the significance of spreading, for example by showing whether Pc-G targets can occur in clusters with common regulation. In addition, transgenic studies will help test whether regions of H3K27me2 correspond with PRE-like elements and whether distinct insulators occur to prevent spreading.

Materials and methods

Plant materials and growth conditions

The *clf-81* mutation (Columbia background) was kindly provided by H Tsukaya. Other Pc-G mutants, reporter lines and growth conditions were as described previously (Goodrich *et al*, 1997; Chanvivattana *et al*, 2004).

Conditional and epitope-tagged CLF lines

To make the conditional *CLF* transgene *pCLF::CLF-GR*, a 7.5k genomic clone that spanned the *CLF* locus was modified by site-directed mutagenesis (Quikchange, Stratagene) to introduce *Bam*HI and *Eco*RI restriction sites immediately 5′ to the stop codon. A *Bam*HI/*Eco*RI fragment of plasmid pBI-ΔGR (gift of A Lloyd) encoding the ligand binding domain of the rat glucocorticoid steroid receptor (residues 508–795) was then introduced as an in-frame fusion at the C-terminus of CLF. The resulting *pCLF::CLF-GR* fusion construct was subcloned into a binary vector conferring glufosinate herbicide resistance *in planta*. Plant transformation of *clf-2/+* heterozygotes by floral dip method gave rise to independent *clf-2 pCLF::CLF GR* homozygotes, all of which showed steroid-dependent rescue of the *clf* mutant phenotype. To make the *35S::GFP-CLF* and *35S::CLF-GFP* constructs, we mutagenised the *CLF* cDNA clone pCDJ4 as described previously (Goodrich *et al*, 1997) to introduce restriction sites 5′ to the start and stop codons, respectively. We then introduced the GFP coding sequences from mGFP6 (gift of J Hasselhof) as in-frame fusions and subcloned the GFP-CLF fusion constructs into pART7/pART27 vectors so as to express them under control of CamV 35S promoter. We transformed *clf-50/+* heterozygotes by floral dip method and thus derived *clf-50* homozygotes that expressed GFP-tagged CLF. At least 10 independent transformants were evaluated for each construct.

Vernalisation treatment

For vernalisation treatments, seeds were surface sterilised, plated on solid MS medium and then kept at 4°C for 40 days. For non-vernalised samples, seeds were similarly treated but kept at 4°C for only 2 days. After moving to long-day conditions (16 h light, 8 h dark, 22°C), plants were grown for 15 days and then harvested.

ChIP and PCR analysis

ChIP was performed exactly as described by Gendrel *et al* (2002) (ChIPs in Figure 5) or with modifications included from Vanoosthuyse *et al* (2004) (Figures 4, 6 and 7). Plants were grown on soil or solid MS medium for 10–14 days, and nuclear enrichment and sonication were performed as described by Gendrel *et al* (2002). Antibodies (2 μg in 100 μl) were incubated for 1–4 h at 4°C with 10 μl of magnetic protein A beads (Dynal) on a roller shaker, which were washed three times with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.0, 167 mM NaCl) before and after incubation with the antibody. After removal of nuclear debris by centrifugation, the sonicated chromatin was diluted 1:10 with ChIP dilution buffer, 1/3–1/4 was added to the antibody-coated beads or beads without antibody (no ab control) and incubated on a roller shaker for 10–15 h at 4°C. Washes were performed as described by Gendrel *et al* (2002), except that only wash with TE was performed. Elution, reverse crosslinking and purification of the DNA were as described by Gendrel *et al* (2002). IP DNA was resuspended in 50–100 μl H$_2$O and stored at −80°C.

IP DNA was analysed by duplex PCR using the primers described in Supplementary Table S1. Reactions were performed in 25 μl with 0.5 μl of IP DNA. PCR conditions were as follows: 94°C 2 min, 35 × (94°C 30 s, 60°C 30 s, 72°C 30 s), 72°C 5 min with varying amounts of primers. The amplified DNA was visualised on 2.5% agarose gels stained with ethidium bromide.

In all ChIP experiments, we amplified DNA using two primer pairs in duplex PCR reactions: one pair was specific for the gene of interest (e.g. *AG*), whereas the other pair was specific for a gene that is not expected to be enriched. Because different primer pairs seldom amplify with equal efficiency in duplex PCR, we compared the relative amounts of the two products amplified from IP DNA against the relative amounts amplified for input (pre-IP) DNA. The ratio reflects the enrichment for a sequence following IP and corrects for any differences in the efficiency of the two primers or of the amounts of chromatin isolated.

Fragment intensity was measured with the program Genetools (Syngene, Cambridge, UK). The relative enrichment was determined as follows: (intensity of band of fragment of interest (in IP)/ intensity of control band (in IP; actin or PFK))/(intensity of band of fragment of interest (in input)/intensity of control band (in input; actin or PFK)). Several dilutions of input were used in amplification to reveal whether amplification was in a linear range. If band

intensity was saturated owing to a high enrichment, the indicated numbers can give only approximate values. If PCR products were present in the 'beads' control, intensities of amplifications were subtracted from the sample (IP with specific antibodies) values.

RT–PCR analysis

Total RNA was extracted with Trizol (Life Technologies) from leaves grown under long-day conditions (16 h light). The integrity of the RNA was verified on a gel before RT–PCR. Reactions were performed in 20 µl with 1 µg of RNA using the ImProm-II RT-System (Promega). Primers were designed to span introns or intron/exon borders to avoid amplification of genomic DNA. Primer sequences can be found in Supplementary Table S1 in Supplementary data.

Confocal microscopy

Plants were grown for 7 days on vertical agar plates containing $\frac{1}{2} \times$ MS and 1% sucrose. Roots were excised 7–10 days after germination, mounted in water and examined using a Bio-Rad Radiance 2100 confocal microscope (Hemel Hempstead, UK). GFP fluorescence was detected with 500-nm long-pass and 530-nm short-pass filters. Images were exported and treated using ImageJ software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Note added in proof

Papp and Muller (*Genes Dev* **20**: 2041–2054) and Kahn *et al* (*J Biol Chem* August 2, 2006) recently described dispersed H3K27me3 methylation at Pc-G targets in *Drosophila*.

Acknowledgements

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