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Antagonistic Roles of SEPALATA3, FT and FLC Genes as Targets of the Polycomb Group Gene CURLY LEAF

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Abstract

In Arabidopsis, mutations in the Pc-G gene CURLY LEAF (CLF) give early flowering plants with curled leaves. This phenotype is caused by mis-expression of the floral homeotic gene AGAMOUS (AG) in leaves, so that ag mutations largely suppress the clf phenotype. Here, we identify three mutations that suppress clf despite maintaining high AG expression. We show that the suppressors correspond to mutations in FPA and FT, two genes promoting flowering, and in SEPALATA3 (SEP3) which encodes a co-factor for AG protein. The suppression of the clf phenotype is correlated with low SEP3 expression in all case and reveals that SEP3 has a role in promoting flowering in addition to its role in controlling floral organ identity. Genetic analysis of clf ft mutants indicates that CLF promotes flowering by reducing expression of FLC, a repressor of flowering. We conclude that SEP3 is the key target mediating the clf phenotype, and that the antagonistic effects of CLF target genes masks a role for CLF in promoting flowering.

Introduction

Plants usually flower at specific times of year, in order to align flowering with periods when pollinators are available and conditions are favourable for growth and fruit set. To achieve this, flowering time is regulated by environmental signals, primarily temperature and photoperiod, and also by intrinsic factors such as the age of a plant. Genetic analysis in Arabidopsis has identified the key components of several flowering pathways, including the photoperiod and vernalization pathways, which mediate responses to daylength and temperature, and the autonomous pathway, which promotes flowering independently of environmental signals [1]. Importantly, the output of these diverse pathways ultimately converges on the control of a few key target genes, termed floral integrators. In addition, genetic analysis suggests that flowering is controlled epigenetically, through factors that act on chromatin of these integrator genes to alter their transcriptional activity. The epigenetic control of flowering is best defined for the vernalization pathway, where long periods of cold such as occur in winter trigger a stable epigenetic change that promotes flowering [2]. In the absence of vernalization, a group of epigenetic repressors termed Polycomb-group (Pc-G) genes play a role in repressing flowering [3,4]. However, the Pc-G regulate genes with opposite effects on flowering, and the relevance of this has not been clear [5].

In Arabidopsis the photoperiod pathway promotes flowering in response to long days. The output of this pathway involves two integrator genes, FT and SUPPRESSOR OF CONSTANS1 (SOC1) both of which promote flowering [6,7]. FT is expressed in vasculature of leaves in long, but not short, days. It encodes a small protein which likely corresponds to florigen, the mobile signal promoting flowering, reviewed recently in [8]. FT protein moves through the phloem from leaves to the shoot apex, where it associates with FD, a HD-ZIP transcription factor, and activates genes such as LFT, promoting floral meristem identity [9]. SOC1 encodes a MADS box transcription factor expressed in the shoot apical meristem and is one of the earliest markers of the floral transition [7,10]. Genetic analysis suggests that FT and SOC1 act in parallel to promote flowering [7]. The vernalization and the autonomous pathways converge on the activity of FLC, which encodes a MADS box transcription factor [11,12]. FLC is a strong repressor of flowering, largely because it binds FT and SOC1 and represses their expression [10]. FLC levels are reduced by the autonomous pathway, which comprises a group of genes with disparate functions. Several members have been found to function in pathways other than flowering. For example, FCA and FPA both encode RNA binding proteins which regulate mRNA 3′-end processing and poly-adenylation of many genes other than FLC [13,14,15,16,17]. Exactly how FPA and FCA reduce FLC activity has been unclear, as both seemingly affect processing of the FLC transcript. However, they were recently shown to regulate the poly-adenylation site selection of antisense FLC transcripts with possible consequences for sense FLC transcription rates [14,15]. In backgrounds with high FLC levels, for example autonomous pathway mutants, vernalization treatment is necessary to reduce FLC activity and permit flowering. FLC levels progressively decline during cold periods [11,12], and are maintained at low levels when plants are returned to warm conditions. The maintenance of FLC
Flowering Regulation through CLF Targets

repression after vernalization is implemented by Polycomb-group (Pc-G) proteins [2].

The Pc-G are a large group of transcriptional repressors which were first identified from genetic screens in Drosophila, on account of their role in regulating homeotic gene expression. Their protein products associate in several complexes, one of which, Polycomb Repressive Complex 2 (PRC2) is widely conserved between animals and plants [18]. Consistent with a role in the epigenetic control of gene expression, the PRC2 has a biochemical activity towards chromatin, specifically catalysing trimethylation of lysine 27 on histone H3 (H3K27me3) [19,20,21,22]. H3K27me3 is correlated with transcriptional repression and to date the PRC2 is the only enzyme known that produce this mark. The catalytic unit of the PRC2 is a SET domain protein first identified as Enhancer of zeste (E(z)I) in Drosophila and represented by three homologues in Arabidopsis, of which only two - CURLY LEAF (CLF) and SWINGER (SWN) - are expressed after germination [23].

Whereas swm mutants are without apparent phenotype, clf mutants are small early flowering plants with narrow, upwardly curled leaves. The clf phenotype is largely caused by mis-expression of the floral homeotic gene AGAMOUS (AG). In wild-type plants, AG is only expressed in flowers where it specifies the identity of stamens and carpels in whorls 3 and 4. In clf mutants AG is expressed outside the flower in vegetative tissues such as leaves and cotyledons. The clf phenotype is largely caused by ectopic AG activity as clf ag double mutants show near wild type leaf morphology and flowering time [3]. Several other genes, including APETALA3, SEPALLATA3 (SEP3), FT and FLC have also been found to be mis-expressed in clf backgrounds but the relevance of this for Pc-G function has not been clear [5,24,25].

We have conducted a genetic screen for modifiers of the clf phenotype and identified suppressors corresponding to fja, sep3 and ft mutant alleles. We show that all three genes are mis-expressed in clf mutants and are direct targets of CLF as their chromatin is enriched for H3K27me3 the levels of which are strongly depleted in clf backgrounds. Genetic analysis indicates that SEP3 mediates the clf phenotype and that FT mis-expression in clf mutants masks a role for CLF in promoting flowering.

Materials and Methods

Plant materials and growth conditions

Plants were grown under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions in controlled environment rooms at 21°C on shelves with fluorescent lighting. Vernalization treatments were performed by sowing seeds on soil, placed in darkness at 4°C for 4 weeks, then transferred to LD or SD conditions at 21°C. The soil used was a mix of Levingtons F2 compost, perlite and sand in proportions 150:60:40. The null clf-50 allele is in Ws background, the clf-28 (Salk 139371) and clf-81 alleles are in Col-0 background [26]. The SEP3::GUS reporter (Col-0) was provided by Dr Hao Yu (Temasek laboratory, Singapore). All other alleles are in Col-0 background. ft-10 and soc1-1 were provided by G. Coupland, fja-7 by G. Simpson, sep3-2 by M. Yanofsky, ftc-3 by R. Amasino.

T-DNA mutagenesis

The T-DNA mutagenesis was performed in the clf-50 pCLF::CLF-GR conditional mutant background in which the clf mutant phenotype is rescued if plants are grown in presence of the steroid dexamethasone [26]. The M0 generation was sprayed with 10 μM dexamethasone every 3 days to provide vigorous, fertile plants suitable for floral dip transformation. The M0 generation was transformed using Agrobacterium strain GV3101 mp90rk carrying the activation tagging construct pJG41, a derivative of pSKI074 [27]. The pJG41 construct carries an extra selectable marker, Ade2::GFP, which renders transgenic seed green fluorescent when viewed under UV illumination [28]. To make pJG41, pSKI074 was cleaved with HindIII and made blunt-ended. The plasmid pFP101 (gift of Francois Parcy) was partially digested with EcoRI, cut with KpnI and a fragment corresponding to the entire pAd23::GFP reporter was gel purified, made blunt-ended and ligated to the linearised blunt pSKI 074. The resulting construct confers both kanamycin resistance and seed fluorescence as independent selectable markers. Primary transformants (M1 generation) were selected on sterile tissue culture medium containing 1/2 MS salts (Duchefa), 0.3% sucrose, kanamycin (50 μg/ml), 10 μM dexamethasone and resistant plants were transferred to soil and sprayed with dexamethasone (10 μM) every 3 days. M2 seed were collected from bulks of two individuals and M2 families were grown on soil without dexamethasone induction and screened for rare families with a suppressed clf phenotype.

Molecular cloning of sequences flanking T-DNA inserts

DNA flanking the T-DNA right border was obtained using the plasmid rescue technique [27]. To isolate DNA flanking the left border, the genome walker PCR technique was used as previously described [29] with the exception that the pSKI074 specific primers Genewalker LB1 5′-GTTTCTGCTATCAAGCCTCCATTGTT-3′ and Genewalker LB2 5′-ACGTGAATGTAGACACGGTCGAA-3′ were used in place of primers LBa1 and LBb1.

Western blot analysis

For anti-FPA western, antibody and protein extraction method were as described [14,30]. For detection of AG, antibody and protein extraction were as described [31]. Separation of proteins by SDS PAGE gel electrophoresis, protein transfer to nitrocellulose membranes and protein detection were performed according to standard procedures.

Gene expression analysis

RNA was extracted from whole seedlings using Qiagen plant RNeasy kits. For first strand cDNA synthesis, 3 μg of total RNA was incubated with 1 μg oligo dT primer (5′-VNTTTTTT-TTTTTTTTTTTTT) at 65°C for 5 minutes in a 10 μl volume, rapidly cooled on ice, then incubated at 42°C for one hour in a 20 μl reaction containing 1× RT buffer (Promega), 1 ul MMLV reverse transcriptase (Promega), 1 ul RNasin (Promega) and 500 uM dNTP. The reaction was terminated by incubation at 65°C for 15 minutes and the cDNA diluted 1/10 with water. Real time PCR analysis was performed using a Roche LightCycler 480 and 10 μl reactions containing 5 μl diluted cDNA, 1× Sybr Green I mix (Roche) and 200 μM primers. Each 10 μl reaction was triplicated (technical replicates) and for each genotype three biological replicates (i.e. independent plant samples) were made. Primer efficiencies were calibrated using a cDNA dilution series and Ct values and relative amounts were determined using the 2nd derivative max method in the Lightcycler 480 software (Roche). Results from different samples were normalised relative to the expression of the Eif4a gene. Primers were as follows: Eif4a 5′-TTCGCTCTTCTTCTTGCTCTG and 5′-GAACGCTAT- CTTGCTCCCTCAAGTA; AG 5′-TCCAGATATAAGCTAAGAGG; SEP3 5′-TATGACGCCTTACAGAGAACC and 5′-ATACCCAT- CGATAAACCTTGTC; SEP1 5′-GCTTAAATGCCATCAACA and 5′-CTAGGACAAGTGGAGATTG; SEP4 5′-ATTTCTCTAACCCTGGCAAGG and 5′-TTCCGTTAATGGAGGT; FLC 5′-CGGTCTCATC-
GAGAAAGCTC and 5'-CCACAAGCTTGCTATCCACA; FT 5'-CTTCAGAGACCTCTATCTTTGGTTATGG and 5'-CTGTTTCGCGACAAGCTGTC.

Chromatin immunoprecipitation (ChIP)

Seedlings were grown for 12 days in sterile tissue culture on MS medium, roots were cut away and the remaining shoots harvested for assay. ChIP assays were performed as in Finnegan et al [32]. Antibodies recognising H3K27me3 (07-449) and H3K4me3 (07-473) were from Millipore. Assays were performed on two independent biological samples with similar results. The relative amounts of DNA in the input, no antibody control and IP samples were quantified by real time PCR using a Roche LightCycler 480 (Roche) as described above, with three technical replicates for each sample. Enrichment was quantified as the proportion of the input DNA that was recovered in the IP sample. The primers used were as follows: AG 5'-CCCAAGGATTTTAGCTGCTCA and 5'-GGTTTCAAGGCGCACTC; FLC 5'-GAGGGCACAAGGAGAACGG and 5'-TGGCCCTTAAACTTATCATCG; SEP3-M 5'-CTTTTGTATTGCTCGGTG and 5'-GAT-GAAATCCGATCCCAAAGT; SEP3-2 5'-GTGTGTTGGTGAGAGTGGAGAT and 5'-ACTGATGAACTCAGACTCAACTAtATACCCC; FT 5'-GTGCTGATCAAGTGCTATCCACA; FUS3 5'-CTGTTGGTGGCATCTTTC and 5'-TAAGCTGGGTTCGTTAAG; SEP1 5'-GTGGCAAGTGTTGATCATGG.

Histochemistry

To assay activity of the GUS reporter gene, whole seedlings were stained, cleared and photographed as described in Chanvivattana et al [23].

Results

A genetic screen for modifiers of the clf mutant phenotype

Because there is considerable redundancy among the closely related Pc-G genes CLF and SWN [23] even null clf mutants have an intermediate level of Pc-G activity; consequently, we reasoned that the clf phenotype would be sensitised to small changes in activity of CLF target genes, for example due to mutations in the target genes themselves or in genes that regulate their activity such as trx-G or Pc-G members. We therefore mutagenised the null target genes themselves or in genes that regulate their activity such as in the autonomous flowering suppressor mutant and found that this harboured a T-DNA insertion in the first intron of SEP3 (Fig. 1B). Consistent with this, the double mutant had greatly elevated FLC levels relative to wild-type and clf-50 plants (Fig. 1B). Although the effects of clf mutations on flowering time are solely due to increased FLC expression [35], FPA is known to regulate many genes other than FLC [13,14]. To test whether the suppression of clf by fpa mutants is solely due to increased FLC activity or rather involves other FPA targets, we made clf-28 fpa-7 ftc-3 triple mutants. This abolished the suppression, i.e the triple mutants had narrow curled leaves like those of clf-28 ftc-3 mutants, suggesting that the suppression of clf by fpa is mediated solely by high FLC levels (Fig. 1B). Lastly, we obtained an additional late flowering suppressor mutant and found that this harbourd a T-DNA insertion in the FCA gene, another member of the autonomous flowering pathway (Fig S2 in supplementary data). Together these results indicate that autonomous pathway mutants suppress clf by causing elevated FLC activity.

The clf phenotype is known to be caused by ectopic expression of AG in leaves of clf mutants [3]. To test whether fpa mutants suppressed the clf phenotype by reducing AG activity, we first measured levels of AG RNA in clf-50 fpa-10 mutants. Unexpectedly, AG mRNA was expressed as strongly in clf-50 fpa-10 as in clf-50 mutants, despite the lack of leaf curling (Fig. 3C). The FPA gene acts by controlling the location within the mRNA of its targets where cleavage and polyadenylation occurs, often leading to changes in the protein product encoded [14]. To test whether the AG protein product was affected by fpa mutation, we analysed protein levels on western blots using a previously isolated antibody to AG protein [31]. We detected two protein products of about 29 Kda that were specific to AG* plants, and these were expressed at a similar level in clf-50 and clf-50 fpa-10 backgrounds (Fig 3D). We concluded that the suppression of clf by fpa mutation occurs independently of AG, and that CLF therefore must have other target genes that are relevant for its mutant phenotype.

fpa mutations suppress clf

We identified a mutant which strongly suppressed the leaf curling and early flowering of clf-50 mutants. The double mutant with clf-50 was also very late flowering relative to the CLF* (Ws) background (Fig. 1A and Fig. 2A). Late flowering mutants can be further characterised by their response to vernalization treatments, which restore normal flowering time to mutants in the autonomous flowering pathway flower but not those in the photoperiodic pathway [33]. The suppressor mutant showed a strong response to vernalization treatments (Fig 2A), suggesting that it affected a gene in the autonomous pathway. Consistent with this, molecular cloning (see materials and methods) revealed that the mutant harboured a T-DNA insertion in the first intron of FPA (see Fig. S1A in supplementary material), a gene acting in the autonomous flowering pathway [34]. The novel fpa allele, designated fpa-10, is likely a null allele as western analysis using an anti-FPA antibody showed that FPA protein, readily detected in wild-type and clf-50 plants, is absent from clf-50 fpa-10 (Fig. 3A). The FPA gene promotes flowering by decreasing expression of FLC, a repressor of flowering [35]. Consistent with this, the clf-50 fpa-10 mutant had greatly elevated FLC levels relative to wild-type and clf-50 plants (Fig. 3B). Although the effects of fpa mutations on flowering time are solely due to increased FLC expression [35], FPA is known to regulate many genes other than FLC [13,14]. To test whether the suppression of clf by fpa mutants is solely due to increased FLC activity or rather involves other FPA targets, we made clf-28 fpa-7 ftc-3 triple mutants. This abolished the suppression, i.e the triple mutants had narrow curled leaves like those of clf-28 ftc-3 mutants, suggesting that the suppression of clf by fpa is mediated solely by high FLC levels (Fig. 1B). Lastly, we obtained an additional late flowering suppressor mutant and found that this harbourd a T-DNA insertion in the FCA gene, another member of the autonomous flowering pathway (Fig S2 in supplementary data). Together these results indicate that autonomous pathway mutants suppress clf by causing elevated FLC activity.

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flowering in Arabidopsis [6,7]. The SOC1 gene carries H3K27me3 methylation [36] and is therefore likely to be a Pc-G target. To test whether SOC1 activity was also required for the clf phenotype, we made clf-28 soc1-1 double mutants. However, the double mutants had similar leaf morphology and early flowering as clf single mutants (Fig. 1E). Thus FT but not SOC1 activity was necessary for the clf phenotype. To test whether the suppression of clf by ft mutation was caused by reduced AG activity, we measured AG RNA levels in clf-50 ft-12 double mutants. However, AG was expressed as strongly in clf-50 ft-12 doubles as it was in clf-50 plants (Fig. 3C), indicating that the suppression was not mediated by AG.

sep3 mutations suppress the leaf curling and early flowering of clf mutations

We identified a third suppressor mutation (Fig. 1F) which, unlike the previous two mutations, had little effect on flowering time. When the mutant was backcrossed to the clf-50, the resulting F1 plants all had a clf phenotype and the F2 generation segregated about 4:1 for the suppressed phenotype (15 in 73 plants), consistent with the suppression being caused by a single recessive mutation. The mutant plants harboured a T-DNA insertion that disrupted both the SEPALLATA3 (SEP3) gene and an adjacent gene of unknown function (At1g24265, see Fig. S1C in the supplementary material). We genotyped ten plants from the segregating F2 and found that the novel sep3 mutation, designated sep3-7, co-segregated with the suppressed phenotype (data not shown). To confirm that disruption of SEP3, rather than the neighbouring At1g24265 gene, suppressed the clf phenotype we created a second sep clf double mutant using the independent sep3-2 and clf-81 alleles in the Col-0 genetic background. Again, a strong suppression resulted (Fig. 1G). Lastly, we recently obtained an additional suppressor mutant with similar phenotype to sep3-7 and found that it contained an T-DNA insertion in the penultimate exon of SEP3 and therefore constituted an independent sep3 allele designated sep3-8 (data not shown). Together these results show that SEP3 activity is required for the clf phenotype.

To test whether sep3 mutations also suppress the early flowering of clf mutants, we measured flowering times in long and short days. The clf-50 sep3-7 plants flowered at the same time as wild-type (Ws) plants in long days and slightly later in short days (Fig. 2C, 41.6 ± 0.78 leaves in Ws versus 44.2 ± 0.93 in clf-50 sep3 see Fig. 2C). Thus SEP3 activity is needed for the early flowering of clf mutants.
To test whether SEP3 might normally have a role in promoting flowering, i.e. in wild-type backgrounds as well as in clf mutant backgrounds, we crossed the sep3-7 clf-50 suppressor mutant to the wild-type Ws progenitor background and screened the flowering time of the resulting F2 in short and long days. About 3/16 of the resulting F2 plants are predicted to be CLF+ sep3-7 genotype, but we did not observe significant differences in flowering time other than some early flowering plants with curled leaves that presumably were clf-50 SEP+ (data not shown). This suggests that SEP3 activity is not needed for normal flowering time.

SEP3 is one of four closely related genes (SEP1-4) that act redundantly and encode co-factors for the activity of AG and other floral homeotic proteins in flowers [37,38]. The suppression of AG-induced leaf curling in clf mutants by sep3 mutations suggests that SEP3 is also needed for AG activity in leaves, but has less redundancy with the other SEP genes in leaves. We therefore measured the expression of the SEP genes in wild-type and mutant seedlings (Fig. 3E). SEP3 expression was indeed strongly upregulated in clf-50 seedlings relative to wild-type (about 400 fold). In addition, when we introduced a SEP3::GUS reporter gene fusion [39] into the clf-50 mutant background, we observed GUS activity in leaves of clf-50 but not wild-type plants (Fig. 3F). By contrast, SEP2 expression showed a slight (three fold) increase in expression in clf-50 mutants (Fig. 3G), and expression of SEP1 and SEP4 was not detectable in wild-type or mutant seedlings (not shown). Together, these results indicated that only SEP3 is strongly misexpressed in clf leaves, so it has less redundancy with the other SEP genes than in flowers, where all four genes are expressed.

Previous studies have shown that the SEP3 gene is required for AG activity in two ways. Firstly, the SEP3 protein is a co-factor needed for AG protein activity [37,40]. Secondly, SEP3 protein can activate AG transcription in flowers [39,41]. To test whether
Figure 3. Gene expression in suppressor mutants. (A) Western blot analysis of FPA protein levels. Three independent clf-50 fpa-10 samples were processed. Note that no protein is detected in the null fpa-7 control, whereas in extracts from a 3SS::FPA-YFP transgenic line a larger product corresponding to the FPA-YFP fusion protein is detected, confirming the specificity of the antibody for FPA. No FPA protein is detectable in fpa-10 extracts, indicating that fpa-10 is likely a null allele. (B) Real time PCR analysis of FLC expression. (C) Real time PCR analysis of AG expression, showing high AG expression in suppressor mutants. (D) Western blot analysis of AG protein expression. The AG antibody detects two proteins of about 29 kDa that are specific for AG, the smaller band possibly representing a truncated product or spurious translation initiation event (Riechmann et al., 1999). AG protein is strongly detected in wild type flowers but not in leaves. Weak expression is found in clf-50 and clf-50 fpa-10 leaves. (E) Real time PCR analysis of SEP3 expression. (F) Histochemical staining of GUS reporter gene activity. SEP3::GUS is not expressed in wild type leaves but shows weak
expression in vasculature of clf-81 leaves (enlarged in inset). (G) Real time PCR analysis of SEP3 expression. (H) Real time PCR analysis of FT expression. (I) Real time PCR analysis of SEP3 expression. Error bars in real time PCR experiments represent standard error of mean of three independent samples (biological replicates). Expression was normalised relative to the Elf4A gene, and is expressed relative to expression in wild type. In B, C, E, G, H whole seedlings less roots of 20 day old short day grown seedlings were used. In I rosette leaves of long day plants at 21 days were used.
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SEP3 has a role in activating AG expression in clf mutants, we quantified AG mRNA levels in seedlings. We found that AG was strongly mis-expressed in clf mutants regardless of SEP3 activity (Fig. 3C). In addition, western blot analysis using an anti-AG antibody indicated that AG protein is present at similar levels in clf and clf sep3 leaves (Fig. 3D). These results suggest that SEP3 is needed for the activity of the AG protein, but not for its stability or for AG transcription in clf mutants.

To test whether AG activity was needed for expression of SEP3 in clf mutants, we measured SEP3 expression in leaves of wild-type, clf-50 and clf-30 ag mutants. SEP3 expression was strongly reduced in clf-50 ag leaves (Fig. 3I). Thus, although AG transcription in clf leaves is independent of SEP3, SEP3 transcription requires AG.

Antagonistic interactions between CLF target genes

Our genetic analysis indicated that in addition to AG, the FT, SEP3 and FLC genes are relevant for the clf phenotype. It is likely that they are direct targets of the Pc-G. All three genes are mis-expressed in clf mutant seedlings (Fig. 3B, E, H). In addition, all three are marked with H3K27me3 methylation, which is characteristic of Pc-G targets [36]. To test whether CLF is required for normal H3K27me3 levels at these genes, we performed ChIP assays using wild-type and clf-50 mutant seedlings. As expected, all three genes were strongly enriched with H3K27me3 relative to a control gene that is not a Pc-G target. In addition, all three genes had reduced H3K27me3 methylation in clf mutants (Fig. 4 and Fig S3 in supplementary data), consistent with their mis-expression in clf. By contrast, the FUSCA3 (FUS3) gene, a Pc-G target which is mis-expressed in clf sun mutants but not clf mutants [42], showed less reduction in H3K27me3 in clf mutants (Fig. 4). To ensure that the reduced H3K27me3 in clf mutants did not simply reflect poor quality extracts from the mutants, we immunoprecipitated the same chromatin extracts using an antibody against the active chromatin mark H3K4me3, and in this case saw increased methylation in the clf mutants (Fig. 4).

The CLF targets have antagonistic effects on flowering, as FT and SEP3 promote early flowering in clf mutants, whereas FLC represses flowering. This suggested that the clf phenotype may represent a balance between these opposing activities. To test this, we first removed FLC activity in a clf background by combining the null flc-3 mutation and clf-28 mutations in a uniform Col-0 background. The double mutants showed stronger leaf curling (Fig. 1H) and much earlier flowering than clf-28 single mutants (Fig. 2D), indicating that FLC indeed antagonises the effects of FT/SEP3/AG on flowering in clf mutants. Secondly, we measured the effects on flowering time of removing FT and FLC activity in the clf background. Strikingly, clf-28 ft-10 mutants were later flowering than ft-10 mutants, despite the fact that clf-28 single mutants are early flowering. By contrast, clf-28 ft-10 flc-3 triple mutants flowered earlier than ft-10 mutants (Fig. 2E). Together, these results show that the effects of elevated FLC expression in clf mutants are masked by increased FT activity – in the absence of the early flowering conferred by FT, increased FLC activity makes clf mutants late flowering.

Mutual activation of SEP3 and FT in clf mutants

Increased expression of FT activates SEP3 in leaves [43] suggesting that the increased SEP3 activity in clf mutants might be due to the increased FT expression. Consistent with this, SEP3 expression was much lower in clf-50 ft-12 mutants than it was in clf-50 mutants, although it was still higher (about 150 fold) than in wild-type (Fig. 3C). By contrast, activation of AG in clf-50 was independent of FT activity (Fig. 3C). We also found that FT misexpression was considerably reduced in clf-50 sep3-7 relative to clf-50 and barely higher (about four fold) than in wild-type

Figure 4. Effect of the clf mutation on histone methylation. ChIP analysis using 12 day old seedlings. Results show H3K27me3 levels at different genes, other than the rightmost bars which show H3K4me3 levels at the AG gene. The SEP3-2 primers amplify a region of the SEP3 promoter previously implicated in regulation of SEP3 by Pc-G proteins [63], SEP3-M amplify part of the large first intron of SEP3. Error bars are standard error of mean of three technical replicates. The experiment was repeated on independent samples with very similar results as shown in supplementary data Figure S3.
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(Fig. 3H). Thus, SEP3 is required for the activation of FT expression that causes early flowering in clf mutants.

An unexpected feature of the suppressor mutants was that although they largely eliminated leaf curling, they showed no reduction in AG activity. However, in all cases FT and SEP3 expression was strongly reduced relative to clf-50, although still slightly higher than in wild-type. This suggested that the suppression of leaf curling is caused by reduced SEP3 and FT expression rather than AG. It is likely that FT activity is required in addition to SEP3 and AG, because in clf-50 ft-12 mutants SEP3 activity is still relatively high compared to wild-type (about 150 fold increased, Fig. 3E) yet leaf curling is suppressed.

Discussion

Whole genome profiling of H3K27me3 suggests that the Pc-G may bind to many thousands of targets in Arabidopsis [36,44]. However, the biological relevance of this binding is uncertain, as a relatively low proportion of the targets are mis-expressed in Pc-G mutants [45]. Previous work showed that AG is necessary for the clf phenotype and that mis-expression of AG causes leaf curling [3,46]. Here, we identify additional mutants that strongly suppress the clf phenotype. Strikingly, the mutants retain strong AG expression in leaves. Our analysis shows that the SEPs FT and FLC genes are also key for the clf phenotype. In particular, we confirm a role for SEPs in promoting flowering, consistent with a previous study showing that SEP3 over-expression causes early flowering [47]. These targets have antagonistic effects on flowering and genetic analysis confirms that the clf phenotype represents a balance of factors promoting and delaying flowering.

High FLC levels suppress clf

Mutations in FPA, which acts in the autonomous pathway promoting flowering, suppress clf. Although FPA is known to regulate genes other than FLC [13,14], our genetic analysis showed that the suppression is caused by the elevated levels of FLC in fpa mutants. Thus, when FLC is inactivated, fpa mutations no longer suppress clf. Despite the suppressed phenotype of clf fpa mutants, they express AG RNA and protein as strongly as do clf mutants. Instead, their levels of FT and SEP3 are strongly decreased. These results are consistent with recent whole genome profiling of sites bound by FLC protein, which showed that SEP3 and FT but not AG are targets [48]. Because the SEP proteins are required for the activity of AG and other floral homeotic proteins [37,38], it is the decrease in SEP3 that is likely most important for suppression of leaf curling in clf backgrounds. SEP3 levels in clf fpa are still higher than in wild-type, which suggests either that there is a threshold of SEP3 activity required for leaf curling and early flowering or that FT activity is also necessary for leaf curling. Consistent with the former, 35S:SEP3 transgenes give variable effects on leaf curling, presumably relating to expression levels [37].

Our results and those of other groups show that CLF represses FLC, so that in clf mutants FLC expression is increased [5,49], albeit much less so than in fpa or fca mutant backgrounds. The relatively minor effects of clf mutation on FLC activity may reflect redundancy between CLF and SWN. Indeed, microarray analysis (data at http://afly.arabidopsis.info/narrays/experimentpage.pl?experimentid=425) shows that clf sun seedlings show much higher increases in FLC expression compared to wild type (118 fold) than do clf mutants (8 fold). It is striking that in the absence of FPA (or FC4 activity, CLF is unable to repress FLC. One possibility is that FPA and FC4 are needed for clf to be recruited to or act on FLC. Previous studies have shown that FPA and FC4 are needed for recruitment of FLD, a H3K4me2 histone demethylase, to FLC [50]. It is possible that the removal of H3K4me2 by FLD is necessary in order for CLF to catalyse H3K27me3 at FLC, for example if H3K4me2 inhibits the H3K27me3 methyltransferase. A recent study shows that H3K4me3 inhibits the activity of a reconstituted CLF/EMFY/MSL1 complex in in vitro assays, and it is plausible that H3K4me2 has a similar effect [51]. There is also a role for COLDAIR, a non cording RNA produced from FLC, in recruiting CLF to FLC [52]. COLDAIR is expressed most strongly during cold treatments, but knock down experiments suggested that it also has a role in recruiting CLF and repressing FLC in the absence of cold treatment [52]. It seems unlikely that FPA and FCA regulate COLDAIR directly via poly-A site selection, as COLDAIR apparently lacks a polyA tail at its 3'-end [52], but might act indirectly via their effects on COOLAIR, the FLC antisense transcript [53].

Activation of SEPs and AG in clf mutants

In flowers, the four SEPs genes largely act redundantly as triple and quadruple knockouts are needed to reveal their function [38,54]. SEP3 has some discrete functions as sep3 single mutants have very subtle effects on petal development [47]; in addition, SEP3 protein shows stronger transcriptional activation activity than the other SEP proteins when assayed in onion cells [37]. In clf mutant leaves, SEP3 is absolutely required for curling, so here there is little redundancy with the other SEP genes. This probably reflects their expression, as (unlike SEP3) SEP1 SEP2 and SEP4 showed little activation in clf. This raises the question of what activates SEP3 in clf mutants. One factor is FT; in 35S-FT plants, SEP3 is expressed in leaves [43], and high levels of SEP3 expression in clf mutants is dependent of FT activity as in clf ft mutants expression of SEP3 is strongly down-regulated. AG activity is also required as in clf ag mutants SEP3 levels are strongly reduced. It is likely that the role of AG is to form an AG/SEP3 complex which autoactivates and stabilises SEP3 expression. This is consistent with microarray analysis of flower development, where transient induction of AG can lead to persistent SEP3 and AG activity via autoregulatory loops in which SEPs/AG complexes bind and upregulate AG and SEP3 [41]. Interestingly, the activation of AG is independent of SEP3 in clf mutant backgrounds. Thus, clf fpa, clf ft and clf sep3 show high AG activity despite low SEP3 levels. This also shows that unlike SEP3, AG does not require FT for its activation in clf leaves.

Role of SEP3 in promoting flowering

Our results show that FT is needed to activate SEP3 in clf leaves, consistent with a previous study showing that over-expression of FT in leaves is sufficient to induce SEP3 expression [43]. Unexpectedly, we also find that SEP3 is required for activation of FT expression in clf mutants. Thus, clf sep3 mutants have low FT levels and flower slightly later than wild-type in short days. SEP3 is therefore needed to promote flowering via FT in clf mutants. This raises the question of whether SEP3 might have any role in promoting the floral transition in wild-type (CLF backgrounds) as well. Precocious expression of SEP3 in leaves using a 35S:SEP3 transgene is sufficient to cause early flowering [37]. In addition, 35S:SEP3-EAR transgenes (which express a fusion of SEP3 to the EAR transcriptional repression domain and presumably inactivate SEP gene targets), confer late flowering in Arabidopsis [55]. However, it is unlikely that SEP3 normally promotes flowering in Arabidopsis: firstly, sep3 mutants showed normal flowering time as in this study and [43]; secondly, SEP3 expression is not detectable in wild type rosette leaves until after the floral transition [43]. However, SEP3 may be important in promoting flowering in other...
species. Thus knockdown of the rice SEP3 homologue delays flowering [56].

Antagonism between targets masks Pc-G role in promoting flowering

The targets of CLF have opposite roles, either promoting (FT, AG, SEP3) or repressing (FLC) flowering and leaf curling. The clf phenotype is therefore a balance of these antagonistic factors. Although Pc-G genes are generally thought to repress flowering, as mutants such as clf and emf2 are very early flowering, they also promote flowering as is revealed by the fact that clf Δt mutants flower later than ft mutants. Such antagonism between targets provides one explanation as to why relatively few predicted targets are mis-expressed in Arabidopsis Pc-G mutants [45], as targets that are repressors may mask the activation of other targets. Similarly, in Drosophila, the activation of several homeobox target genes in Pc-G mutant wing cells prevents the activation of another target, Distal-less (Dll) so that effects on Dll expression are only visible in mutant backgrounds lacking activity of both the Pc-G and the antagonistic homeobox genes [57].

It is also clear that for Pc-G targets such as FT, repression is rapidly overcome during floral induction, for example if short day grown plants are shifted to long days or if the upstream regulator CONSTANS (CO) is induced using a steroid dependent 35S:CO-GR transgene, FT is activated within one day or two hours, respectively [58,59]. Similarly, repression of SEP3 by CLF is overcome in 35S:FT transgenic plants that overexpress FT, although normal expression levels of FT in long day grown plants are insufficient to overcome Pc-G mediated repression in leaves, at least until late in development [43]. In several other cases it has also been shown that Pc-G mediated repression in plants is relatively easily overcome and mainly affects the dynamics of gene expression rather than providing an insurmountable block [32,60,61]. Alternatively, FT SEP3 FLC, and AG, which are normally activated during adult plant development may differ from other Pc-G targets (e.g FUS3), which are permanently repressed after seed maturation, in Pc-G dependent chromatin modifications other than H3K27me3 [62].

Supporting Information

Figure S1 Molecular structure of suppressor mutants. We isolated the DNA flanking the T DNA insertion causing the suppressor mutation using plasmid rescue and genome walker procedures (see methods). The structures shown are the most straightforward interpretation of the data but more complex arrangement are possible, for example tandem T-DNA insertions. Exons are shown as light blue boxes, start of transcription indicated with an arrow. (A) fpa-10 allele. Recovery by plasmid rescue of an EcoRI fragment containing the T DNA right border indicated that the T-DNA insertion was located in the first intron of FPA. (B) An EcoRI fragment containing the T-DNA left border and plant flanking sequences was recovered by the genome walker procedure. Sequence analysis revealed that the T DNA is inserted in the FT first intron. (C) sep3-7 allele. Recovery of an EcoRI fragment by plasmid rescue indicated that the T DNA insertion at At1g24265 is associated with a deletion in the neighbouring SEP3 gene. PCR analysis of genomic DNA confirmed that independent sep3-7 mutants carried a deletion within this region of the SEP3 locus (not shown). (TIF)

Figure S2 Molecular structure of fca-8902 allele. Exons are shown as light blue boxes, start of transcription indicated with an arrow. (A) fca-8902 allele. We recovered a Vphi fragment and a HindIII fragment both containing T-DNA left border and plant flanking sequences. Sequence analysis of these fragments suggests a tandem insertion of at least two T DNAs in inverse orientation within the eighth intron of the FCA gene. The FCA gene produces several transcripts, the gene structure for the beta (functional) transcript is shown (B) Suppression of the early flowering and leaf curling phenotype of clf by fca mutation. Long day plants 21 days after germination (dag). (C) 9 week old plants grown in long days, showing the late flowering phenotype of clf-30 fca-8902 double mutants. (TIF)

Figure S3 Effect of the clf mutation on histone methylatation. ChIP analysis using 12 day old seedlings. Results show H3K27me3 levels at different genes, experiment was performed on independent samples from those in Figure 4. Error bars are standard error of mean of three technical replicates. (TIF)

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Author Contributions

Conceived and designed the experiments: MLV RM JG. Performed the experiments: MLV SY RM FT EDI JG. Analyzed the data: MLV RM JG. Contributed reagents/materials/analysis tools: EDL SY. Wrote the paper: MLV RM JG.

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