Tissue-Specific Expression of FLOWERING LOCUS T in Arabidopsis Is Maintained Independently of Polycomb Group Protein Repression

Sara Farrona,a Frazer L. Thorpe,b Julia Engelhorn,a Jessika Adrian,a,1 Xue Dong,a Liron Sarid-Krebs,a Justin Goodrich,b and Franziska Turcka,2

a Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany
b Institute for Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JH, United Kingdom

The Polycomb Group (PcG) pathway represses transcription through a mechanism conserved among plants and animals. PcG-mediated repression can determine spatial territories of gene expression, but it remains unclear whether PcG-mediated repression is a regulatory requirement for all targets. Here, we show the role of PcG proteins in the spatial regulation of FLOWERING LOCUS T (FT), a main activator of flowering in Arabidopsis thaliana exclusively expressed in the vasculature. Strikingly, the loss of PcG repression causes down-regulation of FT. In addition, our results show how the effect of PcG-mediated regulation differs for target genes and that, for FT expression, it relies primarily on tissue differentiation.

INTRODUCTION

Two main complexes are involved in the repression mediated by the Polycomb Group (PcG) pathway. Polycomb Repressive Complex2 (PRC2) tri-methylates lysine 27 of histone 3 (H3K27me3) and recruits PRC1, which further contributes to the repression of target genes (Hennig and Derkacheva, 2009; Simon and Kingston, 2009). The expression of homeotic (Hox) genes is the paradigm of PcG regulation in animals. Hox genes are expressed only in specific regions of the animal body; however, in PcG mutants their expression extends to other regions, causing homeotic changes in body patterning (Simon and Kingston, 2009). In plants, the best known example of a Hox gene spatially regulated by PcG proteins is the Arabidopsis thaliana MADS box gene AGAMOUS (AG). In the wild-type plants, AG is expressed only in the flowers, whereas in PcG mutants, it is ectopically expressed in leaves and other parts of the plant, correlating with a decrease in H3K27me3 (Goodrich et al., 1997; Chanvivattana et al., 2004; Schubert et al., 2006; Calonje et al., 2008). H3K27me3 target genes in Arabidopsis often show marked tissue-specific expression patterns (Turck et al., 2007; Zhang et al., 2007). Two recent tissue-specific, genome-wide distribution studies of H3K27me3 have revealed that the H3K27me3 is mostly absent from targets in the tissues where these are expressed (Weinhofer et al., 2010; Lafos et al., 2011). On the other hand, changes in H3K27me3 levels are not always correlated with changes in transcription (Schubert et al., 2006; Schwartz and Pirrotta, 2007; Kwon et al., 2009; Adrian et al., 2010).

H3K27me3 is widely dispersed across the FLOWERING LOCUS T (FT) locus (Turck et al., 2007; Zhang et al., 2007), and the histone mark is proposed to delimit the accessibility of transcription factors to certain cis-elements at the FT promoter (Adrian et al., 2010). Chromatin-mediated repression is required to confer photoperiod-dependent control of FT expression (Goodrich et al., 1997; Takada and Goto, 2003). FT encodes a plant florigen and is induced by long-day conditions through the activator CONSTANS (CO) (Turck et al., 2008; Imaizumi, 2010). CO has been shown to bind DNA sequences specifically in vitro, and CO consensus binding sites within the proximal FT promoter are functional cis-elements required for FT expression (Adrian et al., 2010; Tiwari et al., 2010). However, these elements and the proximal promoter alone are not sufficient to drive expression of reporter genes in transgenic plants, but require the presence of enhancer elements that are located more than 4 kb from the transcription start (Adrian et al., 2010). One striking aspect of FT transcriptional regulation is its spatial pattern: prior to flowering, FT is expressed only in the phloem companion cells of the cotyledons and the distal blade of rosette leaves (Takada and Goto, 2003; Yamaguchi et al., 2005; Adrian et al., 2010); after flowering, the gene is also expressed in other organs (cauline leaves, sepals, and fruits) but is still strongly limited to the vascular tissue (Adrian et al., 2010). FT expression is limited to the vasculature even if CO is provided ectopically (Yamaguchi et al., 2005; Adrian et al., 2010). In plants carrying mutations in LIKE HETEROCHROMATIN PROTEIN1 (LHP1), a FT expression pattern similar to that of CO-overexpressing plants is observed (Takada and Goto, 2003). LHP1 directly represses FT as part of a plant PRC1, but in lhp1 mutants the PcG pathway is only partially affected, and the level of H3K27me3 at FT does not change (Turck et al., 2007; Farrona et al., 2008); therefore, the analysis...
of this mutant is not sufficient to understand the role of PcG proteins in FT regulation. As neither CO nor LHP1 are responsible for the characteristic tissue-specific expression pattern of FT, other regulatory components must be involved.

Here we investigate whether the H3K27me3 mark is required for the tissue-specific expression pattern of FT as part of a PcG-mediated repression mechanism that is independent of LHP1. We show that the loss of PcG repression differentially affects targets, and that the domain of FT expression depends on tissue differentiation, even in the absence of a PcG-mediated chromatin configuration.

RESULTS

FT Expression Is Strongly Reduced in the swn-7 clf-28 PcG Mutant

To analyze PcG-mediated FT spatial expression, a transgene carrying an 8.1-kb FT promoter fragment fused to the β-Glucuronidase gene (FTprom:GUS) (Adrian et al., 2010) was introduced into the single clf-28 (clf) and double swn-7 clf-28 (swn clf) PcG mutants (Figure 1). CURLY LEAF (CLF) and SWINGER (SWN) are part of the histone methyltransferase SET family responsible for H3K27me3 deposition. CLF and SWN show partially overlapping functions and are involved in FT repression (Jiang et al., 2008; Liu et al., 2010). In the wild-type plants, the FTprom;GUS signal was obtained only at the vascular tissue of cotyledons and leaves, as expected (Figures 1A and 1D) (Takada and Goto, 2003; Adrian et al., 2010). In clf mutant plants, the signal was stronger (Figures 1B and 1E), which supported the quantitative RT-PCR (qRT-PCR) data (Figure 2A) (Barrero et al., 2007; Jiang et al., 2008), but it was still limited to the vascular tissue. Mutant seeds of swn clf germinate, giving rise to a small seedling that degenerates to a callus-/embryo-like structure (Chanvivattana et al., 2004). In swn clf seedlings, the FT signal was observed at the veins of cotyledons and in leaf-like structures (Figures 1C and 1F). In addition, the roots are also stained, and a strong, diffused signal is observed at the hypocotyls. Strikingly, as the tip of the root develops a swollen and opaque embryo-like tissue, known as a pickle (pkl)-like root phenotype (Ogas et al., 1997), FT promoter-driven signal completely disappears (Figure 1F). In 2-month-old swn clf callus, the GUS signal strongly decreased and was obtained only in a punctate pattern (Figure 1G). Expression

Figure 1. FT Spatial Expression Changes in the Wild Type, clf, and swn clf.

(A) to (G) Histochemical localization of GUS activity in 10- and 20-day-old wild-type, clf, and swn clf seedlings and 2-month-old swn clf callus carrying an FTprom;GUS construct. Ten-day-old wild type (A), clf (B), and swn clf (C); 20-d-old wild type (D), clf (E), and swn clf (F); 2-month-old swn clf (G). Arrow in (F) indicates the loss of GUS signal in the pkl-like root.
analysis confirmed a reduction in FT in swn clf (Figure 2A) despite a maintained expression of CO (Figure 2B). By contrast, AG is strongly expressed in clf and swn clf mutants, indicating that its regulation depends mostly on CLF; whereas STM is up-regulated only in the swn clf background and, therefore, is more dependent on SWN (Figures 2C and 2D) (Schubert et al., 2006).

To further characterize the expression pattern of other PcG targets and to investigate whether other genes mimic FT expression in PcG mutants, the expression of H3K27me3-enriched genes in the wild type, clf, and swn clf was analyzed by expression microarray analysis and clustered according to the different patterns of expression. Although an important percentage of PcG targets was strongly up-regulated in swn clf plants, the clustering showed that FT, which is placed in cluster 1, is not an exception among PcG target genes, because 9.7% of all H3K27me3-enriched genes follow a similar pattern of expression. In addition, other clusters showed only slight expression changes between the wild-type and mutant plants (Figure 3; see Supplemental Figure 1 and Supplemental Data Set 1 online).

In summary, despite the general role of PcG in establishing a repressive chromatin configuration and thereby participating in tissue-specific gene regulation, loss of PcG repression is not synonymous with transcriptional activation, and no general rule predicts how expression might change in PcG mutants.

**FLC Overexpression Is Not the Cause of FT Repression in the swn clf Mutant**

The MADS box transcription factors FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP) directly repress FT (Li et al., 2008), and the expression of FLC itself is regulated by CLF and SWN as part of different PRC2 complexes (Hennig and Derkacheva, 2009; Liu et al., 2010). Like FLC, the SVP locus is covered by the H3K27me3 mark, indicating repression by PcG proteins, although such regulation has not been described (Zhang et al., 2007).

FLC was up-regulated in clf plants compared with the wild-type plants (Figure 2E) (Jiang et al., 2008) and was further increased in swn clf plants, indicating that FLC repression depends on both PcG proteins (Figure 2E). By contrast, SVP is not up-regulated in clf or swn clf plants (see Supplemental Figure 2A online). AP2-like proteins TEMPRANILLO1 (TEM1), TEM2, and SCHLAFMUTZE (SMZ) repress FT expression (Imaizumi, 2010) but are not marked by H3K27me3 and are down-regulated in swn clf (see Supplemental Figures 2B to 2D online). Therefore, FLC, but neither SVP nor TEM1 and 2 or SMZ, might cause FT down-regulation in swn clf. To investigate the role of FLC, FT expression was analyzed in the triple mutant swn-7 clf-28 flc-3 (swn clf flc). Although loss of FLC caused an increase in FT expression in the clf mutant, no up-regulation was observed in
the swn clf background (Figure 2F), demonstrating that FLC is not involved in FT down-regulation in this genetic background.

Chromatin Changes at FT Are Not Sufficient to Explain Its Down-Regulation

FT expression data obtained in clf and swn clf mutants were correlated to changes in FT chromatin (Figure 4A). It has been reported that H3K27me3 is absent at FT in clf (Jiang et al., 2008); however, our ChIP data show that this loss is not homogeneous for the whole FT locus, because significant enrichment of H3K27me3 can still be detected at the 3' region. In swn clf plants, the H3K27me3 signal completely disappeared (Figure 4B). LHP1 binding to FT followed almost exactly the same pattern as H3K27me3 in the single and double mutants (Figure 4C). Similar results were obtained for the AG locus (see Supplemental Figure 3A online).

Loss of H3K27me3 and LHP1 promotes a more permissive chromatin structure, which cannot explain the down-regulation of FT observed in swn clf. Therefore, we analyzed whether, in the absence of H3K27me3, the repressive histone marks H3K9me2 and H3K27me1 were recruited to FT. H3K9me2 ChIP data at FT showed some increase of this mark only in swn clf plants. However, the H3K9me2 increase at FT seems nonsignificant when compared with levels at the Ta3 retrotransposon, although no studies have shown threshold levels for effective H3K9me2-mediated repression (Figure 4D). For H3K27me1, the ChIP data showed an inverse correlation between changes in this mark and H3K27me3 changes at FT (Figures 4B and 4E). An increase in H3K27me1 was also observed at AG, despite the strong up-regulation of this gene in swn clf (see Supplemental Figure 3B online).

H3K4me3 has been shown to act as a bivalent mark together with H3K27me3 at the FT and FLC loci (Jiang et al., 2008; Carles and Fletcher, 2009). Increased H3K4me3 levels were reported in the 5' region of FT and AG in the clf mutant (Carles and Fletcher, 2009). Our ChIP experiments did not show any significant changes for H3K4me3 in the clf background either at FT or AG (see Supplemental Figures 3C and 3D online). An increase for this mark was observed in swn clf plants, despite the repression of FT (see Supplemental Figure 3D online).

In conclusion, despite their divergent expression, FT and AG are subjected to similar changes in chromatin conformation in the PcG mutants. Therefore, chromatin changes are not sufficient to explain FT down-regulation in swn clf.

FT Expression Is Strongly Reduced upon Loss of Vascular Identity in Induced Callus

In swn clf plants, development of the vascular tissue is particularly affected. The characteristic reticulated pattern of the veins is lost in the leaf-like structures, and the linear pattern is

Figure 3. K-Means Clustering of Expression Data of H3K27me3-Enriched Genes in PcG Mutant Seedlings.

The expression data of the H3K27me3-enriched set of genes (n = 4599) were analyzed by K-means clustering (k = 8) using the software Genesis. Expression values for each gene were calculated as fold change compared with the wild type (WT). Clustering was performed on log2-transformed fold-change values. The Pearson correlation coefficient was used as a distance measurement to cluster according to patterns rather than absolute values. Each graph represents one cluster. Squares represent the average expression, and bars indicate the normalized SD. FT is located in Cluster 1.
interrupted in the roots (Figure 1G; see Supplemental Figure 4 online). To analyze the importance of vascular development in the down-regulation of \( FT \) in \( swnclf \) calli, the \( FT_{prom}:GUS \) line in the wild-type background was germinated on callus-inducing medium. In hormone-induced callus, \( FT \) was expressed only in the cotyledons (Figure 5A), which are formed during embryogenesis and were therefore less affected by the hormone treatment. In these organs, \( FT \) was strongly expressed in the veins from where the signal diffused to the surrounding cells (Figure 5B). In the hormone-induced opaque embryo-like tissue that lacked differentiation of veins, the GUS signal disappeared, similar to the results obtained in pkli-like roots and callus of the \( swnclf \) mutant (Figures 1F, 1G, and 5A). The loss of GUS signal in the hormone-induced callus occurred despite a high and ectopic up-regulation of \( CO \) (Figure 5C) and the presence of CO protein (see Supplemental Figure 5 online). Strong expression of \( CO \) in the cotyledons might cause high \( FT_{prom}:GUS \) signal in these organs in the callus-inducing medium.

We next assessed whether an increase in the number of leaf veins could lead to higher expression of \( FT \). \( 1-N \)-Naphthylphthalamic acid (NPA) affects vascular patterning by inhibiting auxin efflux (Wenzel et al., 2008). NPA treatment increases the number and width of vascular bundles but impairs vein differentiation, because the cells within the vascular strand are improperly aligned and the petiole vessels are not connected to those of the stem (Wenzel et al., 2008). \( FT \) expression decreased in NPA-treated plants, despite the apparent increase in vasculature (Figure 6).

**Figure 4.** Chromatin Changes at \( FT \) in the Wild Type, \( clf \), and \( swnclf \).  
(A) Genome browser view of the \( FT \) locus at chromosome 1. Exons of \( FT \) and upstream gene \( FAS1 \) are represented as blue boxes, and untranslated regions are represented as white boxes. Positions of amplicons used in the ChIP analysis are presented as black boxes and are numbered.  
(B) \( \text{H3K27me3} \) at \( FT \) locus. ChIP experiments were performed with chromatin from 10-d-old (10d) and 20-d-old (20d) wild type (wt), \( clf \), and \( swnclf \) and 2-month-old (2m) \( swnclf \) plants. Signals detected along the \( FT \) locus were normalized against the input.  
(C) Binding of LHP1 at \( FT \) locus. ChIP experiments were performed with chromatin from 20-d-old (20d) wild type, \( clf \), and \( swnclf \) and 2-month-old (2m) \( swnclf \) plants carrying a \( \text{35S}_{prom} \text{LHP1:HA} \) transgene. Normalization as in (B).  
(D) \( \text{H3K9me2} \) at \( FT \) locus and the \( Ta3 \) retrotransposon. Data are based on the same chromatin extract and analysis as used in (B).  
(E) \( \text{H3K27me1} \) at \( FT \) locus. Data are based on the same chromatin extract and analysis as used in (B).

Error bars represent \( \pm \text{SE} \) of the mean based on three technical replicates. Dashed lines represent background calculated as average of qPCR signals obtained from each sample with \( \text{Ta3} \) primers ([B] and [C]) or \( \text{RBCS1A} \) primers ([D] and [E]), as described in Methods. A biological replicate of the analysis is included as Supplemental Figure 7 online.
moderate up-regulation in almost 50% of the targets, include genes with a very strong to seedlings. Cluster 2, Cluster 4, Cluster 5, and Cluster 8, with tightly regulated by different flowering pathways (Turck et al., 2008; Imaizumi, 2010). TEM1 and TEM2 are partially overlapping AP2-like transcription factors that repress FT directly by binding to the region encoding the 5’ untranslated region (Castillejo and Pelaz, 2008). SMZ, which belongs to a different AP2 clade (Aukerman and Sakai, 2003), also represses FT expression but interacts with a region several kilobases downstream of the coding region (Mathieu et al., 2009). The MADS box factors FLC and SVP form a complex that represses FT transcription, interacting with CARG cis-elements located in the first intron and FT proximal promoter region (Searle et al., 2006; Li et al., 2008). TEM1, TEM2, and SMZ are not PcG targets, and the microarray analysis showed that these genes are not up-regulated, but rather are down-regulated in swn clf plants. Therefore, we discarded them as the potential cause for FT down-regulation in swn clf plants. SVP is enriched in H3K27me3 but belongs to Cluster 3, which includes genes that are down-regulated in clf and swn clf plants. The results were confirmed by qRT-PCR, indicating that SVP is probably not involved in FT down-regulation in swn clf mutants.

FT is the best characterized PcG target in Arabidopsis. Different PcG complexes have been shown to regulate FLC. The VRN-PRC2 complex participates in the vernalization pathway that stably represses FLC after a prolonged exposure to cold temperatures, whereas the EMF-PRC2 complex seems to regulate FLC independently of vernalization (Hennig and Derkacheva, 2009). CLF and SWN are components of both complexes (Hennig and Derkacheva, 2009; Liu et al., 2010). The expression analysis indicated an additive effect of both proteins in FLC regulation, and, therefore, FLC was a candidate for FT down-regulation in swn clf. However, the genetic data concluded that FLC is not the factor responsible for the decrease in FT expression in the strong PcG mutants. Our results do not exclude that other putative repressors could down-regulate FT in swn clf plants, but the known direct repressors do not have a role in FT down-regulation in the absence of PcG regulation.

**Chromatin Changes Are Not Key Factors in Determining FT Spatial Expression**

The PcG pathway modifies chromatin conformation, which is believed to repress transcription by interfering with the binding of

**DISCUSSION**

**FT Expression Domain Is Reduced in Strong PcG Mutants**

Previous studies have shown the requirement for PcG repression to define territories of expression of different target genes in animals and plants. Consequently, in PcG mutants the expression of such target genes extends to other domains (Goodrich et al., 1997; Chanvivattana et al., 2004; Schubert et al., 2006; Calonje et al., 2004; Schubert et al., 2006; Calonje et al., 2004; Bratzel et al., 2010). We have focused on FT, a PcG target with a well-known and defined spatial pattern, to characterize this regulation further. Although in clf mutants, FT is up-regulated (Figure 2A) (Barreiro et al., 2007; Jiang et al., 2008), our results showed that FT expression in clf is confined to the vascular tissue, its normal domain of expression. However, in calli of the strong PcG double mutant swn clf, FT domain of expression is not ectopically extended, but rather is strongly reduced to a small group of cells. Clustering analysis of PcG targets divided them in eight different clusters according to their pattern of expression in the wild-type, clf, and swn clf seedlings. Cluster 2, Cluster 4, Cluster 5, and Cluster 8, with almost 50% of the targets, include genes with a very strong to moderate up-regulation in swn clf plants, as expected after the loss of a general repressive pathway. Some targets differentially depend on CLF and SWN (Figure 2B) (Schubert et al., 2006). Indeed, Cluster 5 includes genes that depend mainly on CLF, and genes in Cluster 2 depend on both proteins, whereas for genes in Cluster 4, the absence of SWN has a strong effect. Strikingly, ~10% of PcG targets show similar regulation as FT, with high expression in clf and low expression in swn clf (Cluster 1). The dependency of FT on signals lost in the de-differentiated swn clf callus may therefore be a model for the regulation of many PcG targets.

FT down-regulation in swn clf could be due to the up-regulation of a repressor. FT plays a central role in flowering and, therefore, is tightly regulated by different flowering pathways (Turck et al.,
transcription factors to cis-elements, affecting the recruitment of the RNA polymerase and blocking transcriptional initiation and/or elongation (Simon and Kingston, 2009). However, transcription also feeds back on chromatin conformation, and it is not yet fully resolved under which conditions the chromatin plays a determining regulatory role in the regulation of individual target genes (Buzas et al., 2011). Therefore, we investigated whether changes in FT and AG chromatin correlated with the transcriptional changes observed in swntf1 plants.

We observed a reduction of H3K27me3 in tf1 at both loci and a complete loss in swntf1, pointing out that a SWN-PRC2 complex is also involved in FT methylation. LHP1, as part of a PRC1, recognizes and binds to its targets by direct interaction with the H3K27me3 mark (Exner et al., 2009). It has been suggested that H3K27me3 contributes to, but is not fully responsible for, PRC1 targeting (Simon and Kingston, 2009). Our data showed that LHP1 binding mirrors H3K27me3 occurrence, and, therefore, that at FT and AG, H3K27me3 acts as obligatory docking site for LHP1.

In the absence of the PRC signatures H3K27me3 and LHP1, other repressive marks could be recruited and spread over the FT locus. In Arabidopsis, H3K9me2 is a repressive mark characteristic of heterochromatic regions that is excluded from H3K27me3-enriched regions (Turck et al., 2007). Although the minimum level of H3K9me2 required to promote

Figure 6. Expression Analysis of FT in the Presence of an Auxin Transporter Inhibitor.
(A) and (B) Histochemical localization of GUS activity in leaves of 25-d-old 8.1kbFTprom::GUS plants grown on GM plates supplemented with DMSO.
(C) Vasculature organization of a 25-d-old leaf grown on GM plates supplemented with DMSO.
(D) and (E) Histochemical localization of GUS activity in leaves of 25-d-old 8.1kbFTprom::GUS plants grown on GM plates supplemented with NPA.
(F) Vasculature organization of a 25-d-old leaf grown on GM plates supplemented with NPA.
(G) qRT-PCRs in plants grown on GM plates supplemented with DMSO or NPA. Data were normalized to PP2AA3. Error bars represent SE of the mean based on three technical replicates.
down-regulation is unknown, the slight increase in this mark observed at FT in swn clf mutants seems nonsignificant when compared with the H3K9me2 levels at the Ta3 retrotransposon. Consequently, we do not favor a role for H3K9me2 in FT down-regulation. H3K27me1 is also considered as a repressive histone mark and is believed to be the precursor for H3K27me3 (Campos and Reinberg, 2009). In Arabidopsis, a decrease in H3K27me3 does not affect nuclear H3K27me1 levels (Lafos et al., 2011). We did observe local increase in H3K27me1, but, considering that similar changes in H3K27me1 were observed at FT and AG (Figure 4; see Supplemental Figure 3 online), this mark neither predicts nor follows the transcriptional state in swn clf plants. Because H3K27me1 reflects the reduced activity of PRCs, it might also be a PRC2 substrate in Arabidopsis.

H3K4me3 is part of the active Trithorax Group pathway, which antagonizes PcG regulation (Köhler and Hennig, 2010). In animal stem cells, active and repressive chromatin marks are present at the same developmental loci and poise their targets for activation or repression during differentiation (Fisher and Fisher, 2011). In Arabidopsis, bivalent chromatin was demonstrated for FT and FLC (Jiang et al., 2008), and a role of bivalent chromatin in fine-tuning FLC and FT expression was suggested (Jiang et al., 2008; Carles and Fletcher, 2009; Jeong et al., 2009). However, genome-wide data in Arabidopsis did not show a general association between H3K4me3 and H3K27me3 (Ha et al., 2011). Despite previous results (Jiang et al., 2008; Carles and Fletcher, 2009; Jeong et al., 2009), our ChIP experiments did not confirm any significant change for H3K4me3 in the clf background either at FT or AG. An increase for this mark was observed in swn clf plants, despite the repression of FT. Lafos et al. have recently shown that H3K4me3 levels increase globally in swn clf calli, which may explain why higher levels were detected at the FT locus (Lafos et al., 2011). In comparison to H3K27 methylation marks, the levels of H3K4me3 at FT and AG were very low (see Supplemental Figure 3 online), which could explain why the number of genes that are detected as positive for the H3K4me3 mark genome-wide does not change in mutant seedlings that have lost PRC2 function (Bouyer et al., 2011).

**Tissue Differentiation Bypasses Lack of PcG Repression**

Considering that chromatin changes are not sufficient to explain FT down-regulation in strong PcG mutants and that vascular tissue is the normal domain of FT expression, its alteration could be the basis for FT down-regulation in swn clf callus. Auxin is key to the development of the vascular tissue, and important genes involved in the biosynthesis, transportation, perception, and signaling of this hormone are among PcG targets (Notaguchi et al., 2008; Lafos et al., 2011; Rizzardi et al., 2011). Several genes that play an important role in the auxin pathway are affected in swn clf (Figure 3; see Supplemental Data Set 1 online) (Lafos et al., 2011). One possible result of misregulating the auxin pathway is the alteration of vasculature development. This hypothesis is supported by the results in hormone-induced calli and in plants grown in NPA-containing medium. In both situations, the development of the vascular tissue is affected; in hormone-enriched medium by reducing the differentiation of the veins, and in the presence of NPA by increasing the amount and distribution of vascular bundles while impairing their integrity (Figures 5 and 6) (Wenzel et al., 2008). Based on microarray studies performed by the AtGenExpress consortium, neither auxin nor NPA affect FT transcription directly (Goda et al., 2008). Therefore, the results confirm the sensitivity of FT expression to the appropriate development and differentiation of the vascular tissue. The absence of proper vasculature development prevents the activation of FT by CO, which is expressed at normal levels in swn clf and is strongly up-regulated in hormone-induced calli.

In summary, our data show that the role of PcG proteins in the regulatory network determining tissue-specific expression is not identical for all PcG-target genes. Although almost one quarter of

![Figure 7](Figure 7. The Impaired Differentiation of Vascular Tissue Bypasses the Lack of PcG Repression.)

(A) In a wild-type (WT) leaf, the FT expression pattern is the result of the interplay among repressors, such as LHP1 and the PRC2 complex, and activators, such as CO. The PcG pathway is not only involved in the direct repression of FT but is also necessary to promote the proper development of the vascular tissue that is essential for FT activation through an unknown “X” factor.

(B) In a swn clf callus, where the differentiation of the vascular tissue is strongly impaired, CO is not able to activate FT expression due to the absence of the vascular “X” factor, despite the loss of PcG repressive marks in the FT chromatin.
PcG targets are strongly up-regulated in the swn clf mutant (Figure 3) and some have been shown to be ectopically expressed in different PcG mutants (Goodrich et al., 1997; Chanvivattana et al., 2004; Schubert et al., 2006; Calonje et al., 2008; Bratzel et al., 2010), the lack of the repressive H3K27me3 mark is not always synonymous with an increase in the expression domain or increased expression levels. This indicates that the lack of chromatin-mediated repression leads to elevated expression only if additional positive regulators are present. Specifically, the spatial expression of FT depends on the proper development and differentiation of the vascular system, and the removal of the PcG-mediated repressive chromatin structure, even in the presence of the activator CO, is not sufficient to overcome this requirement (Figure 7).

METHODS

Plant Materials and Growth Conditions

All the plants used in this work were the Columbia (Col-0) ecotype. The Plant Materials and Growth Conditions section contains detailed information on the cultivation of these plants.

Methods

Gene Expression Analysis

Total RNA was extracted using the RNeasy mini kit (Quiagen). Five micrograms of RNA were treated using the DNA-free kit (Ambion) to cDNA synthesis. Real time-qPCR was performed using a BioRad iQ5 apparatus and SYBR Green II detection. Expression of PP2A A2 (At1g13320) (Hong et al., 2010) was used for normalization. Primer sets can be found in Supplemental Table 1 online. GST staining was performed as previously described (Adrian et al., 2010).

Microarray Analysis and Clustering of Expression Data

For genome-wide distribution of H3K27me3 in the ecotype Col-0, the data set described elsewhere (Göbel et al., 2010; Reimer and Turck, 2010) was used. H3K27me3-positive regions (chrs) were determined with the implementation of RINGO (Toedling et al., 2007) in the R package ChiPR (half-window-size: 100, dist_CUT_off:200) and were subsequently mapped to genes according to the TAIR8 genome annotation (minimal number of probes per gene: 3). Genes were considered as H3K27me3 targets if at least 20% of the gene or 500 bp were covered by a positive signal. For microarray expression analysis, total RNA was extracted from 10-d-old Col and clf-28 and 12-d-old swn-7 clf-28 seedlings grown on 0.5× MS medium. Affymetrix ATH1 arrays were hybridized with two biological replicates per genotype and processed using the MAS 5.0 procedure (NASCARRAYS-425). For each gene, mean fold-change values compared with Col-0 were calculated. Clustering analysis was performed with all H3K27me3 target genes that were present in the expression data sets (4614 genes).

Clustering was performed with the software Genesis (Sturn et al., 2002). As a distance measurement, the Pearson correlation coefficient was used after applying log2 fold changes using the wild type as reference. For an overview on the major groups of patterns in the data set, hierarchical clustering was performed on a randomly selected subset of 500 H3K27me3 target genes and revealed eight major groups of expression patterns (see Supplemental Figure 1 online). Therefore, k = 8 was used for the K-means clustering analysis shown in Figure 3.

Chromatin Immunoprecipitation

ChIP experiments were performed as previously described (Searle et al., 2006) with anti-H3K27me3 (07-449; Millipore), anti-H3K9me2 (pAb-060-050; Diagenode), anti-H3K27me3 (pAB-045-050; Diagenode), anti-H3K4me3 (ab1012; Abcam), and anti-HA (H6908; Sigma-Aldrich). A small aliquot of untreated sonicated chromatin was used as the total input DNA. qPCR data were normalized against the input and represented as means of three technical replicates. For background, qPCRs with Ta3 primers were used for H3K27me3, H3K4me3, and LHP1:HA ChIPs, and the average of the signal obtained in each sample was plotted (dashed line in graph). A similar method was used for H3K9me2 and H3K27me1, but with RBSC1A primers (a highly expressed gene) as background measure. Primer sets can be found in Supplemental Table 1 online.

Vascular Staining

Staining of the vasculature was performed by incubating ethanol-clarified plant tissues for 30 min in Basic Fuchsin (0.05%) after 10 min of incubation in 0.2 M NaOH. Excess stain was removed by rinsing in lactic acid.

Immunoblotting

Eleven-day-old 35Spro:CO (Jang et al., 2008) seedlings were ground in lysis buffer (50 mM MES, pH 8.5, 25 mM KCl, 5 mM MgCl2, 5% Suc, 30% Glycerol, 10 mM β-mercaptoethanol, 1 mM DTT, 0.3% Triton X-100, 1:1000 Proteinase Inhibitor Cocktail [PIC, Sigma-Aldrich], and 1 mM PMSF). Nuclei were filtered through Miracloth, washed with wash buffer (6 mM MgCl2, 33 mM KCl, 17 mM HEPES, pH 7.5, 1.3% Suc, 13% glycerol, 13 mM β-mercaptoethanol, 1 mM DTT, 1.5 mM PMSF, 0.3% Triton X-100, 1:500 PIC), and resuspended in 500 μL Talon buffer (300 mM NaCl, 50 mM NaPO4, 6 M guanidine HCl 100 μM ZnSO4, 100 mM DTT). Proteins were precipitated by addition of ethanol and guanidine to final concentrations of 95% and 0.3 M guanidine, respectively, and were resuspended in 25 μL of resuspension buffer (50 mM Tris HCl, pH 7.5, 150 mM KCl, 10 μM ZnSO4) and 25 μL of 2× Laemmli buffer. Immunoblots were developed with primary antibodies anti-CO (Valverde et al., 2004) and anti-Histone 3 (ab1791, Abcam) as loading control, and secondary anti-Rabbit IgG-HRPO conjugated (71-035-152, Dianova). The membrane was incubated with a mix of SuperSignal West Dura Chemiluminescent Substrate and SuperSignal West Femto Chemiluminescent Substrate (Pierce Chemical Co.), and the positive signals were detected by a cooled-charge-coupled device camera detection system.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data libraries under the following accession numbers: AG (At1g18960), CO (At5g15840), FLC (At5g10140), FT (Atg65490), LHP1 (At5g17690), SMZ (At5g54990), SVP (Atg62540), TEM1 (Atg25560), TEM2 (Atg68840), CLE (Atg26380), SWN (Atg20202), VIN3 (At5g37980), STM (Atg625360), RBSC1A (Atg67090), GSE20256.
ChIP-chip data). (Polycomb mutant seedlings microarrays), and E-MTAB-749 (H3K27me3 ChIP-chip data).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Representative Example of Hierarchical Clustering Based on Expression Data of a Subset of 500 Randomly Selected H3K27me3 Genes.

Supplemental Figure 2. Analysis of SVP, TEM1, TEM2, and SMZ Expression in the Wild Type, clf, and swn clf.

Supplemental Figure 3. LHP1:HA Binding and H3K27me1 Changes at AG and H3K4me3 Changes at FT and AG in the Wild Type, clf, and swn clf.

Supplemental Figure 4. The Vascular Tissue Loses Its Reticulated Pattern in swn clf.

Supplemental Figure 5. CO Protein Is Stable in Hormone-Supplemented Medium.

Supplemental Figure 6. Biological Repeat of Expression Data Presented in Figure 2.

Supplemental Figure 7. Biological Repeat of ChIP Data Presented in Figure 4.

Supplemental Table 1. List of Primers.

Supplemental Data Set 1. Expression Clusters of H3K27me3-Enriched Genes by K-Means Clustering.

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AUTHOR CONTRIBUTIONS

S.F., F.L.T., J.G., and F.T. designed research; S.F., F.L.T., J.E., and X.D. analyzed data; and S.F. and F.T. wrote the article.

L.S.-K. performed research; S.F., F.L.T., J.G., and F.T. designed research; S.F., F.L.T., J.A., and X.D. analyzed data; and S.F. and F.T. wrote the article.

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