Inheritance of vernalization memory at **FLOWERING LOCUS C** during plant regeneration

**Miyuki Nakamura and Lars Hennig***

Department of Plant Biology and Linnean Center for Plant Biology, Swedish University of Agricultural Sciences, PO Box 7080, SE-75007 Uppsala, Sweden

* Correspondence: lars.hennig@slu.se

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**Abstract**

Specific gene states can be transmitted to subsequent cell generations through mitosis involving particular chromatin (epigenetic) states. During reproduction of plants and animals, however, most epigenetic states are reset to allow development to start anew. Flowering is one of the critical developmental steps by which plants acquire their reproductive capacity. This phase transition is controlled by environmental signals and autonomous regulation. The **FLOWERING LOCUS C** (**FLC**) gene is a flowering repressor that is epigenetically silenced after long-term exposure to cold, ensuring flowering in the spring season. In *Arabidopsis thaliana*, epigenetically silenced **FLC** expression is reset during sexual reproduction. Plants have a remarkable potential to regenerate from somatic cells. However, little is known about whether the regeneration process is similar to sexual reproduction in terms of affecting chromatin states. Here, we tested whether **FLC** silencing is reset during *in vitro* regeneration. Transcriptional repression and high H3K27me3 at **FLC** were both stably transmitted, resulting in early flowering in regenerated shoots. Thus, the silenced epigenetic state of **FLC** is reset only during sexual reproduction and not during *in vitro* regeneration. In contrast, the active epigenetic state of **FLC** was only partially maintained through *in vitro* reproduction, suggesting that regeneration causes stochastic **FLC** silencing.

**Key words:** *Arabidopsis thaliana*, chromatin, development, epigenetic inheritance, phase transition, shoot regeneration.

**Introduction**

Some gene activity states established during development are transmitted to subsequent cell generations through mitosis, often involving particular chromatin (epigenetic) states. Polycomb Group (PcG) proteins are a well-known group of chromatin modifiers that are involved in the maintenance of developmental gene repression in animals and plants, usually through trimethylation at lysine 27 of histone H3 (H3K27me3) at target genes (Geisler and Paro, 2015; Mozgova and Hennig, 2015).

The flowering repressor **FLOWERING LOCUS C** (**FLC**) in *Arabidopsis thaliana* is a well-studied plant PcG protein target (Bastow *et al.*, 2004; Sung and Amasino 2004). **FLC** delays flowering by repressing activators of flowering such as **FLOWERING TIME** (**FT**) (Helliwell *et al.*, 2006; Searle *et al.*, 2006; Geisler and Paro, 2015). The prolonged cold of winter leads to reduced **FLC** expression (Sheldon *et al.*, 2000; Mozgova and Hennig, 2015). Because **FLC** is kept inactive by PcG proteins even when temperatures rise again (Gendall
et al., 2001; Helliwell et al., 2006), flowering can occur rapidly, allowing plants to use the favorable conditions of spring for reproduction. The increase in competence to flower brought about by prolonged exposure to cold is called vernalization (Chouard, 1960).

During reproduction, most epigenetic states on genes are reset to allow development to start anew (Paszkowski and Grossniklaus, 2011). FLC expression, for instance, is reset during reproduction and each generation has to be vernalized to repress FLC for accelerating flowering (Sheldon et al., 2008; Yun et al., 2011). In addition to sexual reproduction, plants have a remarkable potential to regenerate from somatic cells. This regeneration often involves a transient dedifferentiation into callus. Although in vitro plant regeneration has been studied for decades, little is known about whether epigenetic marks are transmitted through callus. For instance, it is not known whether epigenetic states are reset, as occurs during sexual reproduction, or whether they are transmitted to the in vitro progeny. Older observations in the biennial plant honesty (Lunaria biennis), the Arabidopsis late-flowering ecotype Pitzal, and the perennial weed chicory (Cichorium intybus L.) suggest that the vernalized state is maintained after in vitro culture (Wellensiek, 1961, 1962; Burn et al., 1993; Demeulemeester and De Proft, 1999), but detailed molecular studies are not available. For instance, it has remained unknown whether FLC expression and H3K27me3 are affected by in vitro regeneration.

Here, we tested whether FLC silencing is inherited through in vitro regeneration. We found that regenerated shoots from vernalized plants flowered earlier than those from non-vernalized plants. Repression of FLC and high H3K27me3 at FLC, which were induced by vernalization, were stably inherited and not generally reset. Interestingly, some regenerated shoots from non-vernalized plants flowered earlier than the other plants in this group. Moreover, regeneration of shoots led to a moderate increase in H3K27me3 at FLC even without vernalization. Thus, callus induction can affect the abundance of H3K27me3 at FLC, and increased H3K27me3 at FLC may be sustained even in regenerated plants.

Materials and methods

Plant materials and growth conditions

A. thaliana (L.) Heynh Col-0 was used. Col FRI−sf2 (FRI+), AT4G00650, was kindly provided by Dr J. Jarillo (Madrid) (Lee and Amasino, 1995). The flc−6 (SALK_41126, AT5G10140) and vin3−5 (SALK_004766, AT5G57380) alleles were described previously (Mylene et al., 2006; Bouveret et al., 2006).

In vitro plant regeneration

Seeds were placed on 0.5× Murashige and Skoog (MS) plates and kept in the dark at 4 °C for 4 days. Non-vernalized parent plants were grown under long-day conditions (LD) of 16/8h (light/dark) at 22 ± 2 °C for 4–5 days. For vernalized parent plants, after being kept in the dark at 4 °C for 4 days, plates containing imbibed seeds were moved to short-day conditions (SD) of 8/16h for germination and kept for 6 weeks at 4 °C in the same conditions. After the vernalization treatment, plants were transferred to LD at 22 °C and grown for a further 4–5 days. Excised roots from 4–5-day-old plants (+6 weeks for vernalized plants) were transferred to callus induction medium (CIM) (MS salts (Murashige and Skoog, 1962), 2-(N-morpholino)ethanesulfonic acid (MES), 1% sucrose, 0.5 µg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (D9011; Duchefa, Haarlem, The Netherlands), 0.1 mg/l ketin (K2751; Sigma, St. Louis, MO, USA) and 0.8% agarose). Root explants were cultured on CIM plates under low-light LD (3 µmol m−2 s−1) for 4–5 days. For shoot induction, root explants that had been cultured in CIM for 4–5 days were transferred to shoot induction medium (SIM) (MS salts, 0.1% MES, 1% sucrose, 0.5 mg/l 6-benzylaminopurine (B9094; Duchefa), 0.125 µM α-naphthalene acetic acid (N9003; Duchefa), 0.75% agarose). After approximately 1 month of culture on SIM plates under low-light LD, newly emerged shoots were isolated and transferred to plates without any plant growth regulators (1× MS salts, 0.1% MES, 2% sucrose, 0.7% agarose). Flowering date was counted from the start of CIM culture of explants.

Chromatin immunoprecipitation and expression analysis

For analysis of H3K27me3 levels of regenerated shoot, root tissues and aerial tissues were harvested from 9–10-day-old seedlings (+6 weeks for vernalized plants) as the controls. Regenerated shoots were harvested 5–6 weeks after the start of explantation. For analysis of H3K27me3 levels of prolonged culture callus, root tissues were harvested from 7–9-week-old plants (+6 weeks for vernalized plants) as the controls. Chromatin immunoprecipitation (ChIP) was performed as described (Shu et al., 2014). Rabbit IgG (#i5006; Sigma-Aldrich, St. Louis, MO, USA), anti-H3K27me3 (#07-449; Millipore, Billerica, MA, USA) and anti-histone H3-CT-pan (#07-690; Upstate/Millipore#07-690) antibodies were used. ChIP was performed in three biological replicates. Enrichment was calculated relative to histone H3 for ChIP analysis. Expression analysis was done as described (Maksimov et al., 2016). Gene expression values are given relative to a PP2a reference gene (AT1G13320). Primers used are listed in Supplementary Table S1 at JXB online.

Results and discussion

Genetic dissection of flowering time in regenerated shoots derived from vernalized plants

Dedifferentiation of plant cells, as occurs during callus formation, causes major chromatin reorganization (Zhao et al., 2001; Williams et al., 2003; Tessadori et al., 2007; He et al., 2012), which could impair the stability of epigenetic states during in vitro regeneration. To probe the stability of the vernalized state during in vitro regeneration in the Arabidopsis reference strain Col-0, we measured the flowering time of plants regenerated from vernalized or non-vernalized Arabidopsis Col-0 parent plants carrying an active allele of the FLC activator FRIGIDA (hereafter, FRI+), which confers vernalization requirement (Michaels and Amasino, 1999).

Roots from FRI+ seedlings grown at 22 °C for 4–5 days followed by 6 weeks at 4 °C were induced to form callus on CIM. Shoot induction was initiated by transferring the tissue to SIM. Although vernalized plants were 6 weeks older than non-vernalized plants, callus induction and shoot regeneration occurred in a similar manner (Fig. 1A). The number of leaves formed before flowering is often used as a measure of flowering time, but because after in vitro regeneration multiple shoots tend to be clustered, leaf number cannot easily be assigned to a particular shoot. Therefore, we used the number of days from transfer to CIM until flowering as a more objective measure of flowering time. After in vitro regeneration,
wild-type plants and vernalized \( FRI^+ \) plants began to flower 3–5 weeks after starting tissue culture (Fig. 1B). Flowering regenerated shoots from vernalized \( FRI^+ \) plants had relatively smaller rosette leaves, which is commonly observed in early-flowering plants (Supplementary Fig. S1A–C). In contrast, non-vernalized \( FRI^+ \) plants flowered considerably later. Although nearly half of the non-vernalized \( FRI^+ \) plants flowered at between 5 and 9 weeks after starting tissue culture, the remaining plants did not flower even at 12 weeks (Fig. 1B). Regenerated shoots of \( FRI^+ \) derived from non-vernalized plants had a typical late-flowering phenotype, for example, having excess rosette leaf growth (Supplementary Fig. S1D–F).

Next, we tested whether the late-flowering phenotype in non-vernalized \( FRI^+ \) plants depends on the major flowering repressor \( FLC \) by using \( FRI^+;flc \) parents. Regenerated plants from vernalized and non-vernalized \( FRI^+;flc \) parents flowered similarly to regenerated plants from the wild-type Col-0, which lacks functional \( FRI \) alleles, or vernalized \( FRI^+ \) parents (Fig. 1B, C). This result indicates that \( FLC \) is required for the late flowering of regenerated plants in the \( FRI^+ \) genetic background.

Flowering is favored not only by vernalization but also by other external and internal cues, including plant age (Wu and Poethig, 2006; Wu et al., 2009). Because vernalized plants were 6 weeks older than non-vernalized plants, flowering of plants regenerated from vernalized parents might be facilitated by age. To test whether age or vernalization contributed more to flowering time after regeneration, we measured flowering time after regeneration from a \( FRI^+;vin3 \) mutant parent, which
lacks the vernalization response but has no known effect on aging (Sung and Amasino, 2004). Similar to shoots derived from non-vernalized FRI+ parents, shoots regenerated from both vernalized and non-vernalized FRI+; vin3 parents flowered late (Fig. 1D). This result indicates that vernalization but not age of parents accelerated flowering after regeneration. We noted that approximately half of the shoots regenerated from FRI+; vin3 parents flowered late (Fig. 1D). This result indicates that vernalization but not age of parents accelerated flowering after regeneration. We noted that approximately half of the shoots regenerated from FRI+; vin3 parents flowered early (5–9 weeks), regardless of vernalization. This was reminiscent of the early flowering of half of the shoots from non-vernalized FRI+ plants.

Taking these results together, the effect of vernalization on flowering was stably maintained through in vitro regeneration of Arabidopsis Col-0 FRI+ and depended strictly on FLC and VIN3.

Maintenance of H3K27me3 on FLC during dedifferentiation and redifferentiation

The stability of the vernalization effect through in vitro regeneration and the requirement for FLC suggested that repression of FLC by vernalization is maintained through regeneration. To test this hypothesis, we measured FLC expression at different stages during regeneration. The regeneration process can be separated into dedifferentiation and redifferentiation, corresponding to callus formation on CIM and shoot generation on SIM, respectively. We used roots cultured for 5 days on CIM as dedifferentiated tissues and regenerated shoots from callus as redifferentiated tissues. All vernalized roots and aerial tissues as well as regenerated shoots from vernalized FRI+ plants had low FLC expression (Fig. 2A). In contrast, FLC expression was high in all samples derived from non-vernalized FRI+ plants. Thus, the activity state of FLC is largely maintained through in vitro regeneration.

H3K27me3 is required for the maintenance of FLC silencing in somatic tissue (Sung and Amasino, 2004), but its stability during in vitro culture and regeneration is not known. In animals, undifferentiated cells or induced reprogrammed cells often have reduced H3K27me3 (Mansour et al., 2012; Zhu et al., 2013). Similarly, some plant PcG target genes show major changes of H3K27me3 levels during callus induction (He et al., 2012). To test whether H3K27me3 levels at FLC change during dedifferentiation and redifferentiation, we performed ChIP. Across the FLC gene body, relative H3K27me3 levels were maintained throughout regeneration in both vernalized and non-vernalized samples (Fig. 2B). H3K27me3 accumulates at the FLC gene with distinct spatial dynamics. During cold exposure, the nucleation region (from the transcription start site to the 1 kb region) and the distal region (near the 4–5 kb region) gradually gain H3K27me3 (Angel et al., 2011). After moving to warm temperatures, H3K27me3 spreads over the entire FLC gene (Angel et al., 2011). In regenerated shoots, a 3 kb region located between the nucleation and distal regions had slightly lower H3K27me3 levels than other regions (Fig. 2B). Given this pattern, the increase in H3K27me3 during in vitro culture might occur through a similar molecular mechanism to that induced by vernalization. Considering that H3K27me3 is a repressive mark, this result is consistent with the observed expression of FLC. Taken together, these results demonstrate that FLC repression and the presence of H3K27me3 are heritable throughout the in vitro regeneration process. In addition, the regeneration process favored some H3K27me3 accumulation at FLC even in regenerated shoots from non-vernalized plants (Fig. 2C).
The histone demethylase EARLY FLOWERING 6 (ELF6) contributes to the epigenetic resetting of FLC during sexual reproduction (Crevillén et al., 2014). We tested whether the lack of epigenetic resetting during in vitro regeneration was caused by lack of expression of ELF6 or its homolog RELATED TO ELF6 (REF6) during in vitro regeneration (Supplementary Fig. S2). However, both ELF6 and REF6 were expressed at similar levels in ovules and in callus samples, suggesting that expression of the two H3K27me3 demethylase genes is not sufficient for epigenetic resetting of FLC. Instead, demethylase targeting or activity might not be appropriate for FLC resetting during in vitro regeneration. Interestingly, REF6 expression increased in callus samples (Supplementary Fig. S2). This is consistent with widespread changes in H3K27me3 during dedifferentiation (He et al., 2012), which, however, seem not to affect FLC.

**FLC is repressed in some shoots derived from non-vernalized parents**

When pooled samples were used for mRNA expression analysis, no obvious suppression of FLC was observed (Fig. 2A). However, about half of the regenerated shoots that were derived from non-vernalized FRI+ parents flowered before 12 weeks after transfer to CIM (Fig. 1B), suggesting that FLC expression may vary among individual regenerated plants from non-vernalized parents. To test this hypothesis, we measured FLC expression in individual regenerated shoots that were either early or late flowering (Fig. 3A). As expected, all shoots derived from vernalized FRI+ parents had very low FLC expression. Late-flowering shoots that were derived from non-vernalized parents had relatively high FLC expression. In contrast, some early-flowering shoots that were derived from non-vernalized parents had FLC expression as low as
shoots from vernalized FRI+ plants (Fig. 3A). This suggested that in vitro regeneration from non-vernalized parents has the potential to stochastically influence FLC expression.

Interestingly, a moderate increase of H3K27me3 at FLC was observed in regenerated shoots that were derived from non-vernalized or vernalized parents (Fig. 2C). Considering the correlation between the stochastic suppression of FLC expression and the partial increase of H3K27me3, the regeneration process could initiate stochastic alteration of the active FLC state through histone modification.

Because some shoots from non-vernalized plants flowered early despite high FLC expression, FLC expression and flowering time were not strictly correlated in regenerated shoots (Fig. 3A). It was possible that flowering despite high FLC levels was caused by independent activity of the floral activator FT. However, we observed a clear anti-correlation pattern between FLC and FT expression in all tested shoots (Fig. 3B). FT was expressed in shoots derived from non-vernalized FRI+ parents only when FLC expression was low. Unexpectedly, most of the early-flowering shoots from non-vernalized parents had low FT expression, suggesting that flowering in these regenerated plants occurred independent of FT. The stochastic suppression of FLC and apparently FT-independent flowering were also observed in FRI+;vin3 (Supplementary Fig. S3A, B). Further work will be needed to elucidate the mechanism of flowering in the presence of FLC after regeneration. Together, these findings indicate that the inactive, but not the active, epigenetic state of FLC is stably retained through in vitro regeneration.

Effect of prolonged in vitro culture on the H3K27me3 level at FLC

Long-term callus culture can induce hormone-independent cell proliferation, a process termed habituation (Gautheret, 1955). In habituated callus, potential epigenetic clonal alterations occur and the transcription levels of epigenetic regulators can change (Binns and Meins, 1973; Pischke et al., 2006). While the 5–7 days of in vitro culture before shoot induction in our original protocol did not affect the inactive epigenetic state of FLC, prolonged in vitro culture might alter the inactive epigenetic state of FLC. To test this hypothesis, we measured H3K27me3 levels at FLC in calli that were cultured for 2, 4, and 6 weeks on CIM. H3K27me3 at FLC was always higher in calli derived from vernalized plants than in calli from non-vernalized plants (Fig. 3C). However, for 4- and 6-week-old calli derived from non-vernalized plants, H3K27me3 levels were slightly but reproducibly higher than in 7-week-old roots, which have the same total age at 22 °C as 6-week-old calli. As a control, we also measured H3K27me3 levels on IAA2, which has high H3K27me3 in leaves but not in callus (Fig. 3C) (He et al., 2012). H3K27me3 signals at IAA2 showed no clear difference compared with IgG controls during CIM culture, suggesting that the IAA2 locus maintained low H3K27me3 levels throughout 6 weeks of in vitro culture on CIM, in contrast to FLC. Thus, callus induction and extended culture on CIM can cause a vernalization-independent increase of H3K27me3 at the FLC locus.

In conclusion, our study reveals that the silenced, H3K27me3-positive epigenetic state of FLC after vernalization, which is reset during sexual reproduction, is stably maintained during in vitro regeneration. In contrast, the active epigenetic state of FLC is only partially maintained through in vitro reproduction and can stochastically convert to the silent state.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Plant morphology of shoots regenerated from FRI+ parents.

Fig. S2. Expression of histone demethylases REF6, ELF6, and FLC during shoot regeneration.

Fig. S3. FLC expression in leaves from FRI+;vin3 regenerated shoots.

Table S1. Primer sequences in this study.

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Author contributions

MN performed the experiments and analyzed data. MN and LH conceived and designed the experiments and wrote the manuscript.

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