FLOWERING NEWSLETTER REVIEW

FLC or not FLC: the other side of vernalization

Cristina Madeira Alexandre and Lars Hennig*

Institute of Plant Sciences and Zurich-Basel Plant Science Center, ETH Zurich, CH-8092 Zurich, Switzerland

Received 19 December 2007; Revised 11 February 2008; Accepted 15 February 2008

Abstract

Vernalization is the promotion of the competence for flowering by long periods of low temperatures such as those typically experienced during winters. In Arabidopsis, the vernalization response is, to a large extent, mediated by the repression of the floral repressor FLC, and the stable epigenetic silencing of FLC after cold treatments is essential for vernalization. In addition to FLC, other vernalization targets exist in Arabidopsis. In grasses, vernalization seems to be entirely independent of FLC. Here, the current understanding of FLC-independent branches of the vernalization pathway in Arabidopsis and vernalization without FLC in grasses is discussed. This review focuses on the role of AGL19, AGL24, and the MAF genes in Arabidopsis. Interestingly, vernalization acts through related molecular machineries on distinct targets. In particular, protein complexes similar to Drosophila Polycomb Repressive Complex 2 play a prominent role in establishing an epigenetic cellular memory for cold-regulated expression states of AGL19 and FLC. Finally, the similar network topology of the apparently independently evolved vernalization pathways of grasses and Arabidopsis is discussed.

Key words: AGL19, Arabidopsis, chromatin, epigenetics, FLC, flowering time, polycomb, PRC2, vernalization.

Introduction

Early observations reported that prolonged exposure to low temperatures can accelerate flowering in a broad range of plant species (for a review, see Chouard, 1960). This effect is termed vernalization, and constitutes a major determinant in the switch from vegetative to reproductive development. For non-perennials in temperate climates, where the winter season lasts for several months, it is crucial that flowering occurs at the appropriate time, such as in early spring when environmental conditions favour reproductive success. In order to cope with this challenge, plants have devised vernalization mechanisms whereby cold is used as an enabling signal to induce the competence to flower.

One of the distinguishing features of vernalization is the uncoupling between stimulus and effect (Chouard, 1960). This uncoupling is both temporal, because often several months separate the initiation of the vernalization response and the actual transition to flowering, as well as developmental, because even imbibed seeds can become vernalized and retain the vernalized state throughout development until the adult phase. Although many of the physiological aspects of vernalization are still elusive, much progress has been achieved recently at deciphering the molecular basis of the underlying cellular-memory mechanism(s).

In Arabidopsis thaliana, the vernalization requirement is largely conferred by the MADS-box gene FLOWERING LOCUS C (FLC) (Michaels and Amasino, 1999; Sheldon et al., 1999), which is in most vernalization-requiring accessions transcriptionally activated by FRIGIDA (FRI) (Napp-Zinn, 1957; Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). FLC then acts both in leaves and in the apical meristem to repress downstream floral integrators such as FT and SOC1, thereby acting as a floral repressor to delay flowering (Helliwell et al., 2006; Searle et al., 2006).

FLC is not exclusively regulated by vernalization (Fig. 1). A substantial number of additional unrelated positive and negative regulators have been described (for reviews, see Simpson, 2004; He et al., 2005; Quesada et al., 2005; Sung and Amasino, 2006; Schmitz and Amasino, 2007). Vernalization acts at the epigenetic level to stably reduce FLC expression (for a review, see Schmitz and...
Amasino, 2007), and this effect overrides other types of regulation such as by the autonomous pathway. Once FLC is repressed by vernalization, it can only be reactivated in the next generation. The mechanism of FLC repression during vernalization has been studied extensively. The most upstream molecular effect of vernalization is induction of expression of the PHD-domain protein VIN3 (Sung and Amasino, 2004). VIN3 is only expressed at low temperatures, and both transcript and protein levels increase gradually with the length of exposure. Once sufficient amounts of VIN3 protein are present, the cold signal is transduced into chromatin modifications at the FLC locus.

It has been reported that VERNALIZATION 2 (VRN2), a homologue of one subunit of metazoan Polycomb-group Repressive Complex 2 (PRC2), is required for epigenetic maintenance of FLC silencing after vernalization (Gendall et al., 2001; Bastow et al., 2004). More recently, a PRC2-like complex including at least the VRN2, FERTILIZATION INDEPENDENT ENDOSPERM (FIE), and CURLY LEAF (CLF)/SWINGER (SWN) subunits has been reported to associate with VIN3 at the FLC locus (Wood et al., 2006). PRC2 complexes are conserved between plants and animals, and usually have a histone methyltransferase activity that deposits histone 3 lysine 27 trimethylation (H3K27me3) marks on target genes (for a review, see Schwartz and Pirrotta, 2007). Increased levels of H3K27me3 can be observed at FLC following vernalization (Shindo et al., 2006; Sung et al., 2006b; Finnegan and Dennis, 2007; Greb et al., 2007). Subsequently, H3K27me3 is thought to recruit additional repressors such as VRN1 and LHP1, which together assist in the maintenance of a stably silenced state (Levy et al., 2002; Mylne et al., 2006; Sung et al., 2006a). Notably, it has long been known that vernalization requires mitotic activity (Wellensiek, 1962, 1964), and a recent report suggests that both H3K27me3 at FLC and FLC repression cannot be efficiently maintained in non-dividing tissue after transfer to ambient temperature (Finnegan and Dennis, 2007).

Much of the knowledge on the molecular basis of vernalization has come from the studies of FLC regulation, and it is established that FLC is responsible for a considerable part of the vernalization response in Arabidopsis. Nonetheless, an FLC-independent vernalization response exists, because flc null mutants still have a vernalization-sensitive phenotype (Michaels and Amasino, 2001; Moon et al., 2005). In the flc null mutant, both FT and SOC1 are up-regulated after vernalization and this suggests that FLC-dependent and -independent vernalization branches share common targets (Moon et al., 2005) (Fig. 1). Here, the current knowledge about the FLC-independent vernalization response is reviewed.

**FLC-independent responses in Arabidopsis**

**FLC-related repressors of flowering**

*Arabidopsis* FLC is a member of a small family of closely related MADS-domain proteins. Besides FLC, this family contains the five MADS AFFECTING FLOWERING (MAF) proteins with 53–87% identity (Bodt et al., 2003; Ratcliffe et al., 2003). FLOWERING LOCUS M (FLM, also called MAF1) is a repressor of flowering (Ratcliffe et al., 2001; Scortecci et al., 2001), and repression of FLM seems to contribute to the acceleration of flowering by elevated growth temperatures (Werner et al., 2005; Li et al., 2006). It is possible that all MAF proteins function as repressors of flowering (Ratcliffe et al., 2003), but this hypothesis needs experimental testing. So far, a role for MAF1 in the vernalization response has not been reported, but the recent findings that MAF1 can be suppressed by vernalization (Sung et al., 2006b) might bring new insights into MAF1 functions.

FLC and some of the MAF genes share several regulators, such as VIP5 and ELF8/VIP6 (He et al., 2004; Oh et al., 2004) or ESD1 (Martin-Trillo et al., 2006). In addition, plants with reduced DNA methylation have reduced expression of FLC and MAF1-5, suggesting that DNA methylation alters the expression of a common trans-acting regulator (Finnegan et al., 2005). By contrast, some other regulators were found to affect only a subset of the FLC/MAF genes (He et al., 2004; March-Diaz et al., 2007), but it is possible that, at least in some cases,
this reflects assay resolution rather than true biological differences. Similar to FLC, MAF genes are usually regulated by vernalization. Vernalization represses MAF1, MAF2, and MAF3, but it induces MAF5 and does not strongly affect MAF4 (Ratcliffe et al., 2003; Sung et al., 2006b). Furthermore, some natural variability occurs in the regulation of MAF expression, because differences between Col and two late-flowering accessions (Pitztal and Stockholm) have been reported (Ratcliffe et al., 2003). Although H3K27me3 has been found to be essential for FLC repression by vernalization, it is not clear whether this histone mark is involved in the regulation of MAF genes as well. Examination of published data about genome-wide H3K27me3 distribution (Zhang et al., 2007) revealed that MAF4 and MAF5 but not MAF1, MAF2, or MAF3 are decorated with H3K27me3 (Fig. 2). However, in contrast to FLC, which is covered over its entire length by H3K27me3, MAF4 and MAF5 carry H3K27me3 only in certain regions. It should be noted that this genome-wide dataset is based on non-vernalized seedlings and that FLC carries considerable H3K27me3 even under these conditions. Because it is possible that after vernalization H3K27me3 marks accumulate also at MAF1–MAF3, more directed experiments are needed to address the potential involvement of this chromatin mark in MAF regulation.

In addition to FLC and MAF1/FLM, mutant studies have so far only revealed a function for MAF2. maf2 mutants flower slightly earlier than wild type, but still retain a normal response to 6 weeks of vernalization (Ratcliffe et al., 2003). However, if plants were submitted to only 10 d of cold, which is insufficient to elicit a vernalization response in the wild type, a significant acceleration of flowering was observed. In fact, in maf2 mutants a 21 d cold treatment can accelerate flowering to a similar extent as an 85 d cold treatment in wild type, suggesting that MAF2 might regulate the delayed establishment of vernalization (Fig. 3). Such a specific function could prevent, for example, a few days of cold in autumn triggering precocious flowering during winter. This induction of flowering by short periods of low temperature seems to be independent of FLC as no significant decrease in FLC expression could be detected after 10 d of cold treatment (Ratcliffe et al., 2003). Furthermore, 35S::MAF2 plants are unable to respond to vernalization due to continuous SOC1 repression, even when FLC expression is normally reduced (Ratcliffe et al., 2003). It is possible that an ancestor of the FLC/MAF family was a repressor of SOC1 and that at least MAF2 and FLC retained this original function. It will be important to establish if MAF3–5 also repress SOC1 expression and flowering.

The flowering promoter AGL24

Arabidopsis AGAMOUS-LIKE 24 (AGL24) and its parologue SVP belong to the ancient StMADS11 clade of MADS-box genes (Bodt et al., 2003). Curiously, the separation of the AGL24/SVP branch involved strong positive Darwinian selection, and the same was observed for FLC-like genes (Martinez-Castilla and Alvarez-Buylla, 2003). Thus it seems that flowering time control is a specialized function acquired separately in different
MADS-box gene lineages, and maintained most likely through its direct impact on plant fitness (Martínez-Castilla and Alvarez-Buylla, 2003).

AGL24 functions as an activator of flowering in response to vernalization (Yu et al., 2002; Michaels et al., 2003). agl24 mutants are late-flowering, and this phenotype is only slightly suppressed by vernalization much as in soc1 mutants. SOC1 and AGL24 activate each other’s expression but can promote flowering independently as well (Yu et al., 2002; Michaels et al., 2003). Furthermore, unlike SOC1, AGL24 is activated following vernalization in an FLC-independent manner.

In addition to its role in flowering-time control, AGL24 has other developmental functions. Over-expression of AGL24 is correlated with several floral abnormalities consistent with a role in establishing inflorescence meristem identity (Yu et al., 2004). In fact, AGL24 is generally expressed in vegetative organs before the floral transition, but gets progressively cleared as floral development proceeds and eventually becomes confined to the two inner whorls of the flower, the carpels and stamens (Michaels et al., 2003; Yu et al., 2004; Gregis et al., 2006). Recently, it has been shown that repression of the flowering time genes AGL24, SVP, and SOC1 by AP1 is an essential step in the establishment of floral meristem identity (Liu et al., 2007). The emerging picture is that the same developmental regulators can assume distinct roles at different moments in the plant’s life cycle.

The flowering promoter AGL19

Recently, another FLC-independent branch of the vernalization pathway in Arabidopsis was identified (Schönrock et al., 2006). Again, the key regulator responding to the vernalization treatment is a MADS-box gene, a close homologue of SOC1 from the TM3-clade—AGAMOUS-LIKE 19 (AGL19).

Initially described as root-specific, a novel role was assigned to AGL19 after it had been found to be involved in controlling the flowering transition. When ectopically expressed, AGL19 is a potent activator of flowering, but unlike AGL24 only mild floral abnormalities were observed. This suggests that AGL19 has a more restricted role in flowering time control. Also, in contrast to AGL24, AGL19 does not affect SOC1 levels, indicating that it probably acts independently of SOC1 (Schönrock et al., 2006). AGL19 and SOC1 share a conserved similar CArG-box (for CC-A rich GG) motif in their upstream regions. FLC binds the SOC1 CArG-box and represses transcription (Hepworth et al., 2002). In AGL19, the CArG-box sequence differs in a nucleotide that is essential for the FLC-binding to the SOC1 promoter (Schönrock, 2006). Thus, it is likely that FLC cannot bind and repress AGL19. Indeed, AGL19 expression levels are independent of FLC. Furthermore, genetic evidence also supports an FLC-independent function of AGL19 because the double mutant agl19 flc has an additive impairment of the vernalization response (Schönrock et al., 2006).

Interestingly, although AGL19 acts as an activator of flowering, it shares a common regulatory mechanism with the flowering repressor FLC. Both are regulated at the chromatin level by vernalization in a VIN3-dependent manner. Chromatin immunoprecipitation assays demonstrated that AGL19 chromatin is enriched in repressive H3K27me3 before, but much less after, vernalization (Schönrock et al., 2006). While FLC is permanently silenced after vernalization, AGL19 is temporarily silenced before vernalization. It has been reported that FLC silencing involves not only H3K27me3 but also repressive H3K9me2 marks (Bastow et al., 2004; Sung and Amasino, 2004). By contrast, H3K9me2 marks could not be found on AGL19 chromatin. In plants, H3K9me2 is usually associated with stable heterochromatic silencing while H3K27me3 is usually associated with more transient euchromatic silencing (Fuchs et al., 2006), and it is possible that the stable FLC silencing involves additional, heterochromatin-like mechanisms not needed for the transient AGL19 silencing.

H3K27me3 is thought to be deposited by PRC2-like complexes, and H3K27me3 at AGL19 is most likely deposited by a complex of EMF2, CLF/SWN, FIE, and MSI1 (Schönrock et al., 2006). By contrast, H3K27me3 at FLC is most likely deposited by a complex of VRN2, CLF/SWN, FIE, and possibly VIN3 (Wood et al., 2006) (Fig. 4). How the same signal, i.e. prolonged cold, can differentially affect distinct PRC2-like complexes is not known. It is possible that VIN3 assists in assembling the VRN2 complex on FLC (Wood et al., 2006). Because VIN3 is needed for AGL19 regulation as well, it would be
highly interesting to investigate whether VIN3 could mediate disassembly of the EMF2 complex associated with AGL19.

Clearly, many details of the cellular memory of vernalization in Arabidopsis still need to be discovered, but it is exciting to see that stable gene repression by PRC2-complexes is a recurrent scheme. Notably, similar to FLC and AGL19, AGL24 is also decorated with H3K27me3 marks, which are most likely deposited by a PRC2-like complex (Fig. 2). The prominent role of PRC2-like complexes and the H3K27me3 modifications raise the question whether other species use similar mechanisms in their vernalization response.

**Vernalization pathways in other species**

Initially, FLC-like genes were believed to be restricted to the Brassicaceae (Becker and Theissen, 2003), but recent work by Reeves et al. (2007) suggests that the strong positive Darwinian selection acting on these genes might have compromised their identification in other species and that the FLC clade actually originated early in the diversification of the eudicots. Reeves et al. (2007) identified the sugar beet (Beta vulgaris ssp. vulgaris) FLC homologue BvFLC. BvFLC is repressed by extended cold and delays flowering when expressed in transgenic Arabidopsis plants. Further research is needed to clarify how important FLC-like genes are for vernalization responses in non-Brassicaceae species.

The process of vernalization was first discovered in grasses, where vernalization is of great agronomic importance (for historical reviews, see Chouard, 1960; Amasino, 2004). Interestingly, many grasses are short-day–long-day (SD-LD) plants that need to be exposed first to short-day photoperiods and subsequently to long-day photoperiods to flower efficiently (Heide, 1994). In many winter varieties, the initial SD treatment can substitute for the effect of prolonged cold on the induction of the competence to flower (McKinney et al., 1935; Evans, 1987).

Much has been learned about the genetics and molecular mechanisms of vernalization responses in grasses (for a review, see Trevaskis et al., 2007a). Work in barley (Hordeum vulgare) and wheat (Triticum aestivum) led to a model of vernalization that includes four central genes: VRN1, VRN2, VRN3, and VRT2, and genetic data strongly substantiate the importance of grass VRN1-3 for vernalization (Fig. 4). Importantly, despite identical names, wheat and barley VRN1 (also called TmAP1/TaVRT-1 and HvVRN1, respectively) and VRN2 (also called TmZCCT1 and HvZCCTa/HvZCCTb, respectively) do not share any sequence similarity with Arabidopsis VRN1 and VRN2. Instead, grass VRN1 is a homologue of the meristem identity MADS-domain protein APETALA1 (AP1) in Arabidopsis (Schmitz et al., 2000; Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; von Zitzewitz et al., 2005), and grass VRN2 shares the CO, CO-like, and TOC1 (CCT) domain with the flowering time regulator CONSTANS (CO) from the photoperiod pathway of Arabidopsis (Yan et al., 2004). Grass VRN3 is a homologue of Arabidopsis FT, a major component of florigen in Arabidopsis (Yan et al., 2006). In grasses, VRN1 and its homologue FUL are thought to be direct activators of flowering (Preston and Kellogg, 2007), and diploid einkorn wheat T. monococcum mutants that lack VRN1 (TmAP1) do not flower (Shitsukawa et al., 2007). The vernalization response in wheat might include yet another gene, VRT2, a homologue of the Arabidopsis flowering-time genes AGL24 and SVP. VRT2 binds to the VRN1 promoter in vitro, and can recruit VRN2 (Kane et al., 2005); VRT2 and VRN2 together can repress VRN1 (TaVRN1) in a tobacco reporter assay (Kane et al., 2007).
Both vernalization and SD photoperiods can repress VRN2 and thus lift VRN1 repression (Yan et al., 2004; Dubcovsky et al., 2006). However, for efficient activation of VRN1, VRN3 is also needed. VRN3 is repressed by VRN2 and activated by LD photoperiods (Yan et al., 2006). According to this model, vernalization or SD photoperiods are needed to repress VRN2 and thus lift the repression from VRN3 and VRN1. If subsequently LD photoperiods are present, VRN3 will activate VRN1 to induce flowering.

In this model, the signalling network of vernalization in grasses has the same topology as the FLC network in Arabidopsis: Two activators (FT/SOC1 and VRN3/VRN1), which transduce the LD signal for induction of flowering, are repressed by a negative regulator (FLC and VRN2) (Fig. 5). The two activators belong to the same protein families in Arabidopsis and grasses, respectively: FT and VRN3 are both Raf kinase inhibitor-domain proteins (Kardailsky et al., 1999; Kobayashi et al., 1999; Yan et al., 2006), and SOC1 and VRN1 are both MADS-domain proteins (Borner et al., 2000; Lee et al., 2000; Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). By contrast, the repressor that is under negative control by vernalization differs: FLC is a MADS-domain protein (Michaels and Amasino, 1999; Sheldon et al., 1999), but VRN2 is a CCT-domain protein (Yan et al., 2004). This reflects the likely evolutionary history of the signalling networks: FT-like proteins seem to be ancient floral activators that transmit day-length signals in Arabidopsis (LD plant), rice (SD plant), and poplar (perennial tree) but function also in tomato (day-neutral plant) (Bohlenius et al., 2006; Lifschitz and Eshed, 2006; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007). It is believed that vernalization requirement developed independently in Arabidopsis and grasses. In the former, a MADS-box transcription factor evolved to repress FT (and SOC1) in the absence of vernalization. In the latter, a CCT-domain protein evolved to repress VRN3 (and VRN1) in the absence of vernalization.

An alternative model suggests that vernalization in grasses acts primarily on VRN1, which represses VRN2 (Fig. 5) (Trevaskis et al., 2006, 2007a, b). Given that FT is a mobile signal and that FLC acts both in the leaves and in the meristem, it will be important to establish in grasses in which organs and tissue VRN1, VRN2, and VRN3 function. In addition, the role of VRT2 and its homologues needs further investigation. Recently it was proposed that barley VRT2-like genes do not participate in vernalization-mediated repression of VRN1 but rather function in a similar way to Arabidopsis AGL24 and SVP to inhibit floral meristem identity (Trevaskis et al., 2007b).

Another open question is the nature of the cellular memory of vernalization in grasses. While chromatin-based mechanisms, which involve PRC2-like complexes, are needed for the maintenance of silencing in the Arabidopsis FLC and AGL19 branches, it is not clear to which extent such mechanisms control vernalization in grasses. Two lines of evidence suggest that this might be the case: First, three wheat VIN3-like (VIL) genes (TmVIL1–3) were described (Fu et al., 2006). Similar to Arabidopsis VIN3, TmVIL1–3 transcripts accumulate after 4–6 weeks of cold treatment, but return rapidly to pre-vernalization levels after the shift to ambient temperatures. It remains to be tested whether VIL proteins mediate the vernalization response in grasses. Second, transcriptional profiling of the vernalization response in perennial rye grass ( Lolium perenne) identified not only a MADS-box gene and a VRN2-like CCT-domain gene but also a JUMONJI (JmjC)-like gene, LpJMJC (Cianna.me et al., 2006). Although JmjC-domain proteins have not yet been found in the Arabidopsis vernalization response, the JmjC-protein REF6 is a repressor of FLC, possibly by modifying FLC chromatin (Noh et al., 2004). LpJMJC is not a close homologue of REF6, but it may also act through chromatin remodelling. This idea is supported by the recent finding that JmjC-domain proteins are often histone-demethylases (Klose et al., 2006). More research is needed to establish whether JmjC proteins such as LpJMJC mediate epigenetic regulation of vernalization-responsive genes in grasses.

Conclusions

The ability to be vernalized is an important adaptive trait in plants. Evolution found multiple answers to the old
question: ‘how to check that the winter is over and that a “better life” can begin?’ (Becker et al., 2003). Mechanisms of FLC-dependent vernalization are understood best, but it remains to be tested how widely they are used outside of the Brassicaceae. At least in grasses a different vernalization pathway evolved. Interestingly, even within a single species, vernalization signalling can follow multiple branches. It is possible that, after the first vernalization pathways evolved, selection favoured the addition of more robust branches to the vernalization signalling network. In the case of Arabidopsis, vernalization involves FLC-, AGL24-, and AGL19-dependent branches, and it is currently not clear which of the three is most ancient and which is most recent. Despite the multiple appearances of pathways for vernalization during evolution, there are recurrent themes. First, vernalization networks consist of similar network motifs and are of similar topology. This suggests that certain network structures evolve easily and give a robust performance. Second, epigenetic memory mechanisms are used both in the FLC- and the AGL19-branches of the Arabidopsis vernalization pathway. Such epigenetic mechanisms ideally serve the purpose of stable maintenance of previously established gene expression states. It will be exciting to see whether epigenetic mechanisms such as PRC2-mediated gene silencing participate in other vernalization pathways as well.

Acknowledgements

We thank Vivien Exner for critical reading of the manuscript, and two anonymous reviewers for their insightful comments. Work in the authors’ laboratory is supported by SNF project 3100AO-116060 and ETH project TH-16/05-2.

References


Bodit SD, Raes J, Peer YV, Theissen G. 2003. And then there were many: MADS goes genomic. Trends in Plant Science 8, 475–483.


Finnegan EJ, Kovac KA, Jaligot E, Sheldon CC, Peacock WJ, Dennis ES. 2005. The downregulation of FLOWERING LOCUS C (FLC) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms. The Plant Journal 44, 420–432.


