International Journal of Plant Developmental Biology ©2007 Global Science Books

FLC-Independent Vernalization Responses

Cristina Alexandre • Lars Hennig*

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

Corresponding author: * **lars.hennig@ipw.biol.ethz.ch**

ABSTRACT

Varieties of many plant species show a requirement for prolonged exposure to low temperatures in order to accelerate flowering, a process termed vernalization. In Arabidopsis, the early-flowering phenotype of vernalized plants results from the combined action of three MADS-domain proteins - FLC, AGL19 and AGL24, each assigned to an independent vernalization pathway. Both AGL19 and AGL24 function to promote flowering, and are activated during vernalization, while FLC acts to delay flowering and therefore is repressed by a vernalizing-treatment. One conspicuous attribute of vernalization is a delayed effect that is coupled to a cellular memory-mechanism. For both FLC and AGL19 pathways this cellular memory has been found to be based on epigenetic modifications. One model is that two distinct histone-modifying Polycomb repressive complexes – the VRN2- and EMF2-complexes - introduce repressive histone H3 lysine 27 trimethylation marks at specific locations in the *FLC* and *AGL19* chromatin, respectively, leading to mitotically stable transcriptional repression. Vernalization acts differentially on each complex, and the coordinated action of both is necessary for a complete vernalization response. As homologs of the Arabidopsis vernalization genes are being identified in other species, it may soon be revealed whether the same mechanisms are shared by distinct plant groups. However, it is believed that vernalization responses evolved independently in different plant groups, and in grasses, epistatic interactions between two loci, *VRN1* and *VRN2* that are unrelated to the Arabidopsis *VRN1* and *VRN2* genes, mainly determine the vernalization requirement. Whether epigenetic mechanisms are also involved in the vernalization response outside Arabidopsis remains to be determined. Importantly, *VRN1* in grasses encodes a MADS-domain protein. Thus, MADS-domain proteins play central roles in various vernalization pathways.

Keywords: *AGL19*, Arabidopsis, chromatin, cold, epigenetics, flowering time
Abbreviations: **AGL**, AGAMOUS-LIKE; **CArG–box**, CC(A/T)$_6$GG motif, MADS domain protein binding element; **ChIP**, chromatin immunoprecipitation; **FLC**, Flowering Locus C; **H3K27met3**, Histone 3 Lysine 27 trimethylation; **LD**, long days; **MADS-box**, DNA-binding domain/transcription enhancer factor; **PcG protein**, Polycomb-Group protein; **PRC2**, Polycomb Repressive Complex 2; **SD**, short days

CONTENTS

INTRODUCTION

Floral initiation, i.e. the differentiation of floral primordia at the shoot apical meristem, commits the plant to flowering and constitutes the first event in flower development. Maximal reproductive success is only achieved if favorable external conditions are present at the time of flowering and seed set. Therefore, an intricate network of regulatory pathways tightly couples transition from vegetative to floral development with environmental signals. Such signals include photoperiod, ambient temperature, extended exposure to low temperatures, light quality and quantity, among others (for review see Mouradov *et al*. 2002). The relative importance of these signals differs between species: photoperiod, for instance, is an essential signal in the long day plant *Nicotiana sylvestris* or the short day plant *Nicotiana tabacum*, var. 'Maryland Mammoth', but is dispensable in the facultative long day plant *Arabidopsis thaliana*. Photoperiod is even irrelevant in day neutral *N. tabacum* cultivars such as 'Wisconsin 38' (for review see Vince-Pue 1994).

For many annual and biannual plants in the temperate zones, extended exposure to low temperatures, as typically

Received: 15 February, 2007. Accepted: 1 September, 2007.

Invited Review

experienced during winter, constitutes a major determinant of floral initiation. The effect of temperature has been discovered in seeds of winter cereals, which must be planted before the end of winter in order to fruit within 12 months of sowing. As early as 1857 Klippart reported results from systematic research on this subject (Klippart 1857). He showed that among the various climatic factors of winter, the determining factor is the cold temperature to which the young plants are subjected for several weeks; this makes the winter cereals capable of flowering soon after the return of warmer temperatures. Seelhorst and Gassner extended this work (Gassner 1918; van Seelhorst 1898), and in 1928 the infamous Russian agronomist Lysenko established that slight imbibition makes the cereal seed susceptible to the action of cold without inducing germination, that otherwise could prevent the use of a sowing machine. As spring cereals are called Jarovoe in Russian (from "Jar": formerly fire, or the god of spring), Lysenko called the process that makes a winter cereal behave like a spring cereal "Jarovization" and translated the word into the English "vernalization" (Latin *vernum* meaning spring) (Lysenko 1928).

Today, vernalization usually refers to "the acquisition or acceleration of the ability to flower by a chilling treatment" (Chouard 1960). This definition emphasizes that vernalization does not directly cause flowering but rather increases plants' competence to integrate other flowering-inducing signals. Both the range of effective temperatures and the required duration of the cold exposure differ between species (for review see Chouard 1960), but the key-point is that prolonged cold exposure is always required. Beyond a threshold exposure the vernalizing effect is quantitative, with increasing periods of cold leading to faster responses, until an optimum is achieved.

The first studies on the mechanism of vernalization responses were performed at the physiological level, either through localized cooling of plant organs or by grafting non-vernalized plants with vernalized ones (for a comprehensive classic review see Chouard 1960). It was observed that several organs are responsive to vernalization, namely excised embryos, leaves and roots, and often can be used to regenerate flowering shoots (Wellensiek 1962; Wellensiek 1964; Metzger 1988). In some cases, grafting of vernalized "donors" caused non-vernalized "receptors" to flower. This was achieved in the dicot black henbane (*Hyoscyamus nigra*), but also demonstrated for sugar beet (*Beta vulgaris* L.), carnation (*Dianthus barbatus*) and pea (*Pisum sativum*) (for review see Lang 1952; Chouard 1960). The grafting experiments were interpreted as evidence for the action of a flowering hormone – *vernalin*. *Vernalin* was considered to differ from *florigen*, the postulated flowering hormone of photoperiodic responses (Melchers 1939). However, graft-transmission of the vernalized state seems to be the exception rather than the rule (Lang 1952; Chouard 1960) and no direct evidence of the existence of a *vernalin*-like compound was ever presented. Additionally, the apical meristem is capable of becoming vernalized independently, and therefore, a clear distinction between the site of production and site of action cannot be made for the vernalizing agent(s) (Lang 1952). It is somehow disappointing that we do not know much more about transmission of vernalization states today than we did 50 years ago.

Vernalization was described as an inductive process, with a delayed effect (Lang 1952). In fact, the cold treatment can be perceived from very early stages, but is only effective upon return to warm temperatures, often several months later. It was readily established that cold-induced changes must be self-perpetuated throughout plant development, and are closely related to cell proliferation. To this respect, Wellensiek (1962, 1964) performed a series of experiments with excised portions of leaves of *Lunaria biennis*, and observed that vernalization was restricted to the neighboring areas of the cut surface. Also in the same work, he found that only young but not old, fully expanded leaves can be vernalized. Wellensiek then concluded that dividing cells are the targets of cold, irrespective of their location *in planta*. The exciting idea suggested by these experiments was that the vernalized condition is linked to DNA replication and is faithfully transmitted through mitosis.

VERNALIZATION PATHWAYS

The *FLC*-dependent vernalization pathway

The development of molecular genetic tools has boosted our understanding of vernalization during the last decade (for recent reviews see Sung and Amasino 2005; Sung and Amasino 2006). Most of the progress in this field has been achieved studying the model plant *Arabidopsis thaliana*. However, even before the spring of molecular techniques, the genetic control of vernalization was addressed in Arabidopsis by crossing summer-varieties, which do not require vernalization to flower, with winter varieties. This led to the identification of two loci deemed essential for conferring a winter-habit: *FRIGIDA* (*FRI*) (Napp-Zinn 1957) and *FLOWERING LOCUS C* (*FLC*) (Koornneef *et al*. 1994; Lee *et al*. 1994). Cloning of the genes revealed that *FLC* encodes a MADS-box transcription factor and *FRI* a transcriptional activator of *FLC* (Michaels and Amasino 1999; Sheldon *et al*. 1999; Johanson *et al*. 2000). Subsequent experiments established that FLC is a central regulator of flowering in Arabidopsis, acting at the convergence of three major flowering pathways (**Fig. 1**): the vernalization and autonomous pathways repress *FLC*; and the photoperiod pathway is down-regulated by FLC via its repressive action on *FLOWERING LOCUS T* (*FT)* and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1)* (reviewed in Boss *et al*. 2004).

Mutant analysis revealed that *FLC* is directly involved in the vernalization response, and furthermore, *FLC* transcript levels were correlated with the duration of the cold treatment and the promotion of flowering (Sheldon *et al*. 2000). In contrast, *FRI* acts only indirectly, by enhancing *FLC* expression to levels which are effective in repressing flowering (Michaels and Amasino 1999; Sheldon *et al*. 1999). In addition to *FRI*, several other *FLC* activators have been identified. Some of them are *FRI*-dependent, like the *VERNALIZATION INDEPENDENT* (*VIP*) genes, which encode subunits of the Paf1-complex. This complex establishes histone H3 lysine 4 trimethylation (H3K4met3) of *FLC* chromatin to allow efficient transcription (He *et al*. 2004; Oh *et al*. 2004). Another activator of *FLC*, PHOTOPERIOD INDEPENDENT EARLY FLOWERING1 (PIE1), is similar to ATP-dependent chromatin-remodeling proteins of the ISWI and SWI2/SNF2 family (Noh and Amasino 2003). Thus, much regulation of the vernalization mediator *FLC* appears to involve chromatin-based mechanisms.

FLC acts as a potent inhibitor of flowering, because it represses transcription of the floral integrator genes *SOC1* and *FT* (Lee *et al*. 2000; Samach *et al*. 2000) that would otherwise activate meristem identity genes such as *APETALA1* (*AP1)* and *LEAFY* (*LFY*) (**Fig. 1**) (for review see Parcy 2005). The mechanism by which FLC represses *SOC1*, has been suggested to involve direct binding to the *SOC1* promoter region harboring a CArG-box motif (MADS domain protein binding element), since mutations in this CArG-box reduced *SOC1* repression in transgenic *35S::FLC* plants (Hepworth *et al*. 2002). ChIP assays have recently revealed that a direct FLC-*SOC1* interaction occurs *in vivo*, and might involve the action of a multi-protein complex (Helliwell *et al*. 2006; Searle *et al*. 2006). Notably, the repression of *FT* by FLC may be spatially confined to leaves, and involve a direct interaction of FLC with the first intron of *FT* (Searle *et al*. 2006). Consequently, it was proposed that FLC has a dual action, both in leaves and in the apical meristem with leaf-produced signals being necessary to give competence to the apical meristem to respond correctly to vernalization (Searle *et al*. 2006). These results shed new light on the problem of long-distance action of vernalization, but still more experimentation is required.

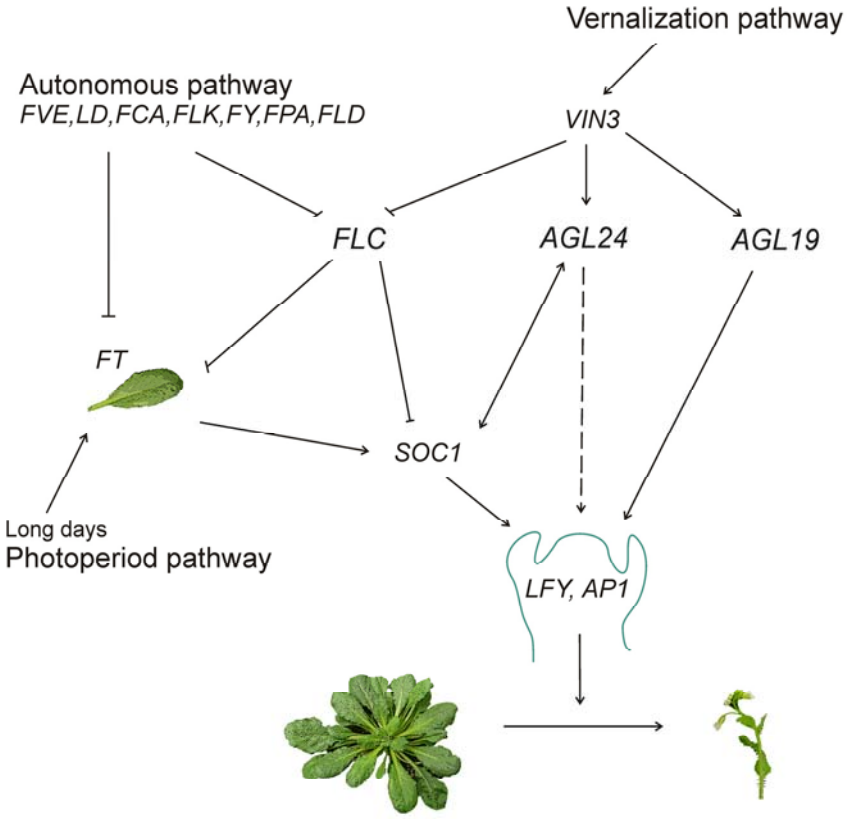

Fig. 1 Vernalization acts through different pathways to promote flowering in Arabidopsis. A single upstream element, *VIN3*, is sensitive to extended cold exposures and initiates the vernalization response. Three MADS-box genes – *FLC*, *AGL19* and *AGL24* – are regulated by *VIN3*, and all contribute to the early-flowering phenotype of vernalized plants. *FLC* is a key-regulator of several flowering-time pathways and acts to delay flowering by repressing *SOC1*, either directly or through *FT* (in the photoperiod pathway). In turn, *SOC1* directs the activation of meristem-identity genes *LFY* and *AP1*. During vernalization, *SOC1* is activated, primarily through repression of *FLC* but also directly by *AGL24*. However, in contrast to *FLC*, *AGL24* does not require *SOC1* to promote flowering, although it is not known if this is a direct effect (dashed line). Interestingly, *AGL19* promotes flowering after vernalization through a completely separate pathway, without affecting *SOC1*. Downstream interacting partners are still unidentified.

Vernalization represses FLC

Vernalization treatments repress *FLC* transcription (Michaels and Amasino 1999; Sheldon *et al*. 1999), and several mutants are known that are impaired in this process. The most upstream element identified so far is *VERNALIZATION INSENSITIVE 3* (*VIN3*) (Sung and Amasino 2004). In *vin3* mutants the vernalization response is completely blocked and *FLC* is not repressed (Sung and Amasino 2004). VIN3 contains a FNIII (fibronectin type III) domain, which is normally involved in protein-protein interactions, and a PHD domain, which is often found in chromatin remodeling protein complexes and can confer binding to H3K4met3 (Mellor 2006). *VIN3* expression is induced by cold, and protein levels accumulate only over an extended period of cold-exposure. Furthermore, *VIN3* expression overlaps with that of *FLC*, but is not sufficient to maintain stable *FLC* repression. This requires the action of at least two other genes – *VERNALIZATION 1* (*VRN1*) and *VERNALIZATION 2* (*VRN2*) (Gendall *et al*. 2001; Levy *et al*. 2002). In both *vrn1* and *vrn2* mutants, *VIN3* expression and at least part of *FLC* repression are still induced by cold, but FLC levels rapidly rise when temperature increases. The current model suggests that *FLC* is initially repressed by VIN3 and that this repressed state is then maintained by VRN1 and VRN2 (Henderson and Dean 2004).

Epigenetic maintenance of FLC repression

The study of VRN2 function greatly benefited from the discovery of the VRN2 homologs Suppressor of Zeste 12 (Su(Z)12) in insects and humans (Birve *et al*. 2001; Koontz *et al*. 2001). Arabidopsis, Drosophila and human, VRN2 and Su(Z)12 are subunits of the Polycomb Repressive Complex 2 (PRC2) (Czermin *et al*. 2002; Kuzmichev *et al*. 2002; Müller *et al*. 2002; Wood *et al*. 2006). In addition to VRN2, the VRN2-complex contains FERTILISATION INDEPENDENT ENDOSPERM (FIE), CURLY LEAF (CLF), SWINGER (SWN) and likely additional subunits, and it can associate with VIN3 in a PRC2-like complex (Wood *et al*. 2006). Insect and human PRC2 have histone methyltransferase activity that introduces H3K27met3 modifications into the chromatin of target genes; such H3K27met3 marks allow binding of additional repressive protein complexes that eventually lead to stable transcriptional repression (for review see Cao and Zhang 2004; Bantignies and Cavalli 2006). In Arabidopsis plants, *FLC* chromatin is enriched in repressive H3K27met2 and H3K9met2, but only if plants were vernalized and have functional VIN3, VRN1 and VRN2 (Bastow *et al*. 2004; Sung and Amasino 2004). Curiously, in *vrn1* mutants H3K27met2 seems mostly normal but is not sufficient to maintain *FLC* repressed, suggesting that VRN1 acts downstream of VRN2-mediated H3K27met2 modifications (Bastow *et al*. 2004; Sung and Amasino 2004; Mylne *et al*. 2006). In parallel to dimethylation marks, vernalized *FLC* chromatin is also decorated with H3K9met3 and H3K27met3 marks (Sung *et al*. 2006b). Because PRC2 catalyzes mainly trimethylation of H3K27, this observation nicely supports the model of PRC2-mediated repression of *FLC*.

Another component that is required for epigenetic regulation of *FLC* is *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*), the plant homolog of HP1 in animals (Gaudin *et al*. 2001). HP1 binds heterochromatic trimethylated H3K9 and contributes to heterochromatin maintenance (for review see Maison and Almouzni 2004), and Arabidopsis LHP1 binds to H3K9met2/3 and H3K27met3 (Turck *et al*. 2007). During vernalization, LHP1 associates with *FLC*, where it remains after return to warmer conditions. In vernalized *lhp1* mutants, both H3K9met2 marks at *FLC* and *FLC* repression are lost (Mylne *et al*. 2006; Sung *et al*. 2006a). These data are consistent with the idea that plant LHP1 mediates stable repression of PRC2 target genes (Turck *et al*. 2007).

A general model for the repression of *FLC* by vernalization is now emerging (**Fig. 2**): initially, *VIN3* expression is induced by lasting cold; VIN3 binds to *FLC* chromatin and recruits histone-deacetylase activity and the VRN2-containing PRC2 complex; the VRN2-complex likely introduces H3K27met3 marks into *FLC* chromatin, which in turn help to recruit LHP1 and also VRN1; LHP1 and VRN1 then assist to establish H3K9met2 marks and together are required to maintain *FLC* transcription shut off.

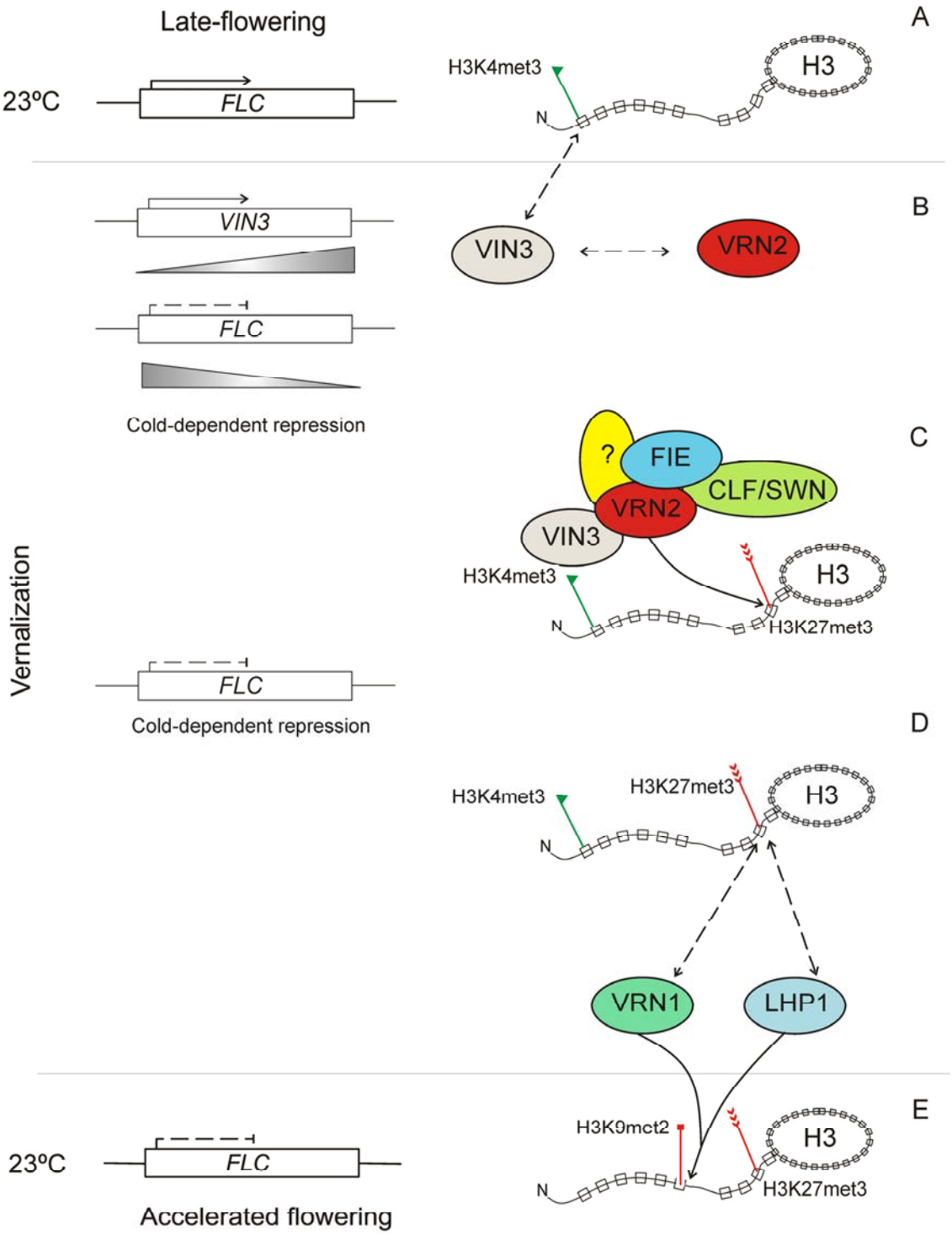

Fig. 2 Vernalization represses *FLC* through epigenetic mechanisms. Without vernalization (A), plants are late-flowering due to active *FLC* transcription. At the chromatin level, the *FLC* locus is enriched in the permissive H3K4met3 marks. During vernalization, *FLC* is gradually repressed likely involving several molecular steps: (B) VIN3 protein accumulates in response to vernalization and contributes to the initial transcriptional inactivation of *FLC*; in addition, VIN3 helps to recruit VRN2 to *FLC* chromatin, possibly through recognition of H3K4met3 marks present in active *FLC*. (C) VRN2 is part of a Polycomb Repressive Complex 2 (VRN2-complex) with histone methyltransferase activity that will tri-methylate lysine residue 27 of histone 3, thereby introducing the first repressive mark on *FLC*. (D) Once established, H3K27met3 serves as downstream recognition signal for other chromatin modifying proteins such as VRN1 and LHP1, which both contribute to subsequently introduce H3K9met2 signals. (E). Finally, *FLC* will be stably repressed, resulting in accelerated flowering. Dashed arrows represent protein recruitment or interaction; solid arrows represent insertion of chromatin modifications. It is not known whether different histone marks such as H3K4met3 and H3K27met3 coexist on the same histone tails in *FLC* chromatin; and various details of this model still await rigorous molecular tests.

FLC-independent vernalization pathways

In Arabidopsis, *FLC* repression is the major mode of vernalization. However, *flc* mutants still respond to vernalization (Michaels and Amasino 2001), and thus *FLC*-independent vernalization pathway(s) exist. In addition, clear *FLC* homologs have not been identified for all plant groups (Becker and Theißen 2003; Reeves *et al*. 2007; **Table 1**), indicating that such *FLC*-independent vernalization pathways might be of major importance in other species.

Table 1 Overview of FLC- independent vernalization-responsive loci.

Species/Loci	Encoded protein (Arabidopsis homologs)	Function	Reference
Arabidopsis			
AGL19	MADS-domain protein (SOC1)	Activator of flowering	[a]
AGL24	MADS-domain protein (SVP)	Activator of flowering	[b,c]
Cereals			
VRN1	MADS-domain protein (AP1)	Activator of flowering	[d,e]
VRN2	CCT domain protein (CO)	Repressor of flowering	[d,e]
ZCCT1, ZCCT2			
VRN3	Homology to RAF kinase inhibitor (FT)	Activator of flowering	[f,g]
VRT2	MADS-domain protein (SVP, AGl24)	Putative repressor of flowering	[h]
Lolium perenne			
LpMADS1	MADS-domain protein (AP1)	Putative activator of flowering	[i]
LpMADS10	MADS-domain protein (SVP)	Putative repressor of flowering	[j]
LpCOL1	CCT-domain protein (CO)		[k]
LpJMJC	JUMONJI-like protein (ELF6, REF6)	Putative chromatin remodeling activity	[k]

[a] Schönrock *et al*. 2006; [b] Michaels *et al*. 2003; [c] Yu *et al*. 2002; [d] Takahashi and Yasuda 1971; [e] Tranquilli and Dubcovsky 2000; [f] Turner *et al*. 2005; [g] Yan *et al*. 2006; [h] Kane *et al*. 2005; [i] Petersen *et al*. 2004; [j] Ciannamea *et al*. 2006b; [k] Ciannamea *et al*. 2006a.

Arabidopsis

The floral activator AGL19

In Arabidopsis, a major *FLC*-independent vernalization pathway is based on the floral activator *AGAMOUS-LIKE 19* (*AGL19*) (Schönrock *et al*. 2006). *AGL19* belongs to the type II class of MADS-box genes and is phylogenetically related to *AGL14* and *SOC1/AGL20* (Becker and Theißen 2003). In contrast to earlier studies, which described *AGL19* as a root-specific gene (Alvarez-Buylla *et al*. 2000), it was recently reported that *AGL19* is also expressed in above-ground organs including rosette leaves and flowers (Schönrock *et al*. 2006). The similarity of *AGL19* to *SOC1*, a well-known floral pathway integrator (Lee *et al*. 2000; Samach *et al*. 2000), led to the investigation of potential roles of *AGL19* in flowering time control. It was found that *AGL19* is a potent activator of flowering if ectopically expressed, but that under standard laboratory conditions, *AGL19* has only a minor role in the promotion of flowering. However, *agl19* mutants can no longer adequately accelerate flowering in response to lasting cold treatments, indicating that this gene has an important role in the vernalization response (Schönrock *et al*. 2006).

How does AGL19 function relate to the *FLC*-dependent vernalization pathway that activates *SOC1*? Both *SOC1* and *AGL19* have increased expression after vernalization and promote flowering by activating meristem identity genes, such as *LFY* and *AP1* (Schönrock *et al*. 2006). Over-expression of *AGL19* in wild-type plants did not affect *SOC1* expression, and over-expression of *AGL19* in a *soc1* background was sufficient to promote flowering, indicating that *AGL19* does not require *SOC1* to function (Schönrock *et al*. 2006). On the other hand, over-expression of *SOC1*, which greatly accelerated flowering, strongly repressed *AGL19*, indicating that negative cross-regulation might be present. Most importantly, *FLC* is not required for repression of *AGL19* prior to vernalization and AGL19 levels do not influence *FLC* expression. Finally, impaired vernalization responses in *agl19* and *flc* are additive in the *agl19 flc* double mutant. Thus, it was concluded that *AGL19* functions in an *FLC*-independent vernalization pathway (see **Fig. 1**) (Schönrock *et al*. 2006). FLC is known to repress *SOC1* expression via binding to a CArG box in the *SOC1* promoter (Hepworth *et al*. 2002). Interestingly, in the *SOC1* homolog *AGL19* this CArG-box is conserved but has a mutation at a position that is essential for *in vitro* binding of FLC to the *SOC1* promoter (Hepworth *et al*. 2002; Schönrock 2006).

Similar to *FLC*, *AGL19* is repressed by a PRC2 complex, but while *FLC* is repressed *after* vernalization by the VRN2-complex, *AGL19* is repressed *before* vernalization by the EMF2-complex (Schönrock *et al*. 2006). This PRC2 complex contains the VRN2 homolog EMBRYONIC FLOWER 2 (EMF2), FIE, CLF and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Hennig *et al*. 2005). *AGL19* repression is lost in *emf2* and *clf* mutants as well as in plants with reduced MSI1 levels due to co-suppression (*msi1-cs*)

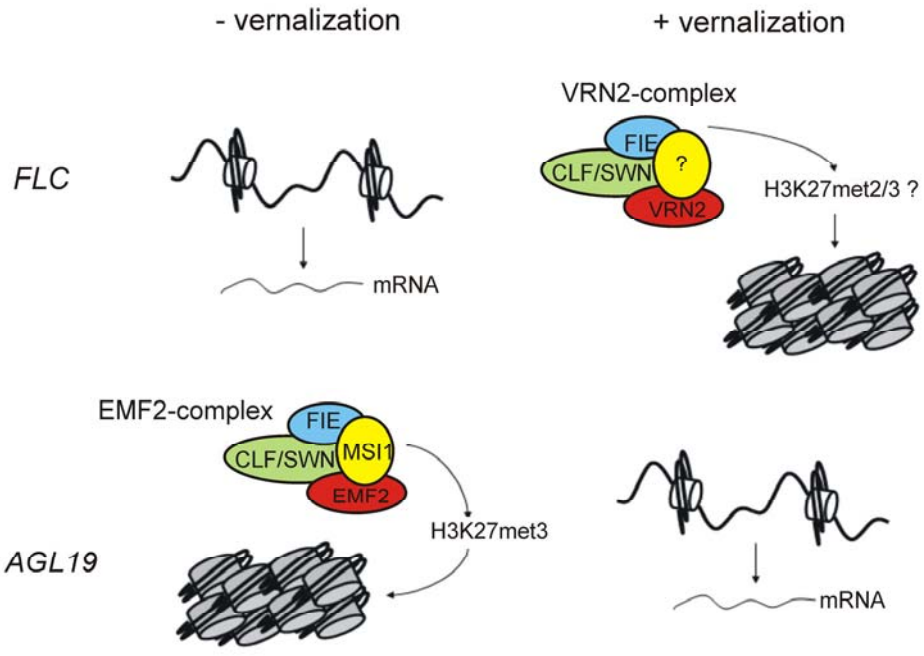

Fig. 3 *AGL19* and *FLC* are regulated by distinct PRC2-like complexes. Persistent repression of *FLC* after vernalization requires the VRN2-complex, which is similar to the metazoan Polycomb Group Repressive complex 2 (PRC2). In addition to VRN2, the VRN2-complex includes FIE and CLF/SWN; it is assumed that this complex has methyltransferase activity targeting histone 3 lysine 27 (H3K27). Current models support the idea that di- and trimethylation of H3K27 influence chromatin dynamics and promote a more compact state that is recalcitrant to transcription. A similar mechanism regulates *AGL19*, but in this case a distinct PRC2-like complex is involved. Two subunits (FIE and CLF/SWN) are conserved between the two complexes, while two others subunits (EMF2 and MSI1) might not be conserved. Because the EMF2-complex target *AGL19* is enriched in H3K27met3, it is assumed that the EMF2-complex has histone methyltransferase activity mediating stable gene repression.

(Hennig *et al*. 2003; Schönrock *et al*. 2006). In contrast, *SOC1* expression was not affected in *clf* or *msi1-cs* plants, suggesting that this complex is unique to the *AGL19* pathway. VIN3 and VRN2 are both needed for *FLC* regulation by vernalization, but only VIN3 and not VRN2 is needed for *AGL19* regulation. *AGL19* chromatin is enriched in H3K27met3 marks, especially at the 5′ region of the gene, and these marks are greatly reduced in both *clf* and *msi1-cs* plants (Schönrock *et al*. 2006). In contrast to *FLC*, however, there was no H3K9met2 detected at *AGL19*. Because much of the H3K27met3 at *AGL19* chromatin disappeared after vernalization, it was concluded that the EMF2-complex, which contains MSI1 and CLF, represses *AGL19* before, but not after vernalization (Schönrock *et al*. 2006). How vernalization overcomes this PRC2-like repression at the *AGL19* locus is completely unknown; one possibility is by preventing the establishment of the H3K27me3 marks in the first place.

FLC repression and vernalization are quantitative responses, which correlate with the duration of cold (Sheldon *et al*. 2000). It needs to be tested whether *AGL19* activation by vernalization has similar quantitative kinetics. Similarly, direct downstream targets and interacting partners for AGL19 are still unknown. However, what is known so far about *FLC* and *AGL19* regulation by vernalization supports a model where vernalization in Arabidopsis involves the coordinated action of two independent PRC2-like complexes: the VRN2-complex, which represses *FLC*, and the EMF2-complex, which represses *AGL19*. Interestingly, the action of these two complexes is inversely affected by vernalization and coordinated in time (**Fig. 3**).

The floral activator AGL24

The *flc agl19* double mutant is still partially vernalization-responsive, demonstrating that in Arabidopsis at least one other pathway mediates vernalization. This pathway likely involves *AGAMOUS-LIKE 24* (*AGL24*), a dosage-dependent promoter of flowering (Yu *et al*. 2002; Michaels *et al*. 2003). Mutants for *AGL24* show a phenotype similar to that of *soc1*, with a normal photoperiod response, but being late-flowering under long-day and short-day conditions (Yu *et al*. 2002; Michaels *et al*. 2003). Unlike mutants from the autonomous flowering pathway, the late-flowering phenotype is not strongly suppressed by vernalization (Michaels *et al*. 2003). Together with genetic and expression analyses, these observations suggest a role for *AGL24* as a positive regulator of *SOC1*. Conversely, over-expression of *SOC1* is also increasing *AGL24* expression (Michaels *et al*. 2003). No such positive cross-regulation exists between *AGL19* and *SOC1* (Schönrock *et al*. 2006). Nonetheless, SOC1 and AGL24 do not require each other to promote flowering (Michaels *et al*. 2003). Both *SOC1* and *AGL24* are up-regulated by vernalization but while for *SOC1* this involves *FLC* repression, it seems to be *FLC*-independent in the case of *AGL24* (Michaels *et al*. 2003). Currently, it is not known how vernalization regulates *AGL24* and whether this involves any chromatin-based, epigenetic mechanisms. It would also be interesting to determine if VIN3, VRN2 or VRN1 are involved in regulation of *AGL24*. This could provide additional evidence for the role of *VIN3* as the cold-responsive element common to all vernalization pathways in Arabidopsis.

In situ localization of *AGL24* transcripts showed that *AGL24* was expressed in the whole zone of the vegetative shoot apical meristem and emerging leaves but as the floral development progressed, the *AGL24* transcripts were found to be restricted to the carpel and stamen primordia (Yu *et al*. 2002, 2004). This expression pattern suggests that *AGL24* might play simultaneous roles in the regulation of flowering time and in the establishment of floral meristem identity (Yu *et al*. 2002). In fact, one of the primary functions of *LFY* and *AP1* in establishing floral meristem identity is transcriptional repression of *AGL24*. Without this repression, extended *AGL24* expression in later developmental stages promotes inflorescence identity and causes floral reversion (Yu *et al*. 2004). Similarly to *AGL24*, *SOC1* and the MADS-box flowering-time gene *SVP* (*SHORT VEGETATIVE PHASE*) are direct targets of AP1 repression in early floral meristem development (Liu *et al*. 2007). Ectopic expression of any of the three genes leads to defects in floral meristem development, resembling the inflorescence-like phenotype of *ap1* mutants.

Other species

Extending our knowledge on the genetic and molecular basis of vernalization beyond Arabidopsis is important, not only from an evolutionary perspective, but also as a potential tool to increase the agronomic value of crop species. Grasses, for example, are mostly grown for their vegetative production, and the controlled inhibition of flowering would result in a significant increase in feed quality (Bruinenberg *et al*. 2002).

A brief overview of current progress in several important species is given below.

Eudicots

Physiology of vernalization is well studied in legumes (Chouard 1960), but molecular data on the vernalization responsive elements is still incipient. Recently, Hecht and colleagues (2005) have identified several equivalents of the Arabidopsis *FLC* regulators, namely *VRN1*, *VERNALIZATION INDEPENDENCE3* (*VIP3*, *VIP4*), *EARLY IN SHORT DAYS4* (*ESD4*), and *PIE1*.

No *FLC* or *FRI* homologues have been identified in pea, and it has been generally accepted that *FLC*-like genes are restricted to the Brassicaceae (Becker and Theißen 2003). However, novel findings indicate that the lineage of *FLC*-homologs originated early in the diversification of the eudicots (Reeves *et al*. 2007). These authors suggested that the strong positive Darwinian selection of *FLC*-like genes (Martinez-Castilla and Alvarez-Buylla 2003) might have prevented the identification of more distant *FLC*-homologs. The first functional study performed in sugar beet (*Beta vulgaris* ssp. *vulgaris*) demonstrated that it's *FLC*-homolog, *BvFLC*, is repressed by extended cold and can function as a flowering repressor when ectopically expressed in Arabidopsis (Reeves *et al*. 2007). This exciting study suggests that *FLC*-like vernalization responses can be shared between a large number of eudicot plants species, and further studies might rapidly change our view on the evolution of vernalization responses.

Grasses

Major bottlenecks of genetic studies of vernalization in cereals are the frequent high genome complexity and the lack of adequate fully sequenced vernalization-sensitive species. Up to now, most studies have focused on winter varieties of wheat and barley.

QTL analyses suggested that mainly two loci – *VRN1* and *VRN2* – account for the vernalization-responsiveness of wheat and barley (Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000), and both loci have been cloned. *VRN1* (also identified as *TaVRT1* or *WAP1*) encodes a MADS-domain transcription factor similar to the Arabidopsis meristem identity gene *APETALA1* (*AP1*) (Schmitz *et al*. 2000; Danyluk *et al*. 2003; Trevaskis *et al*. 2003; Yan *et al*. 2003; von Zitzewitz *et al*. 2005). Natural allelic variation at the *VRN1* locus correlates with different vernalization requirements in wheat (Yan *et al*. 2003). Plants with dominant *VRN1* alleles flower early without the need for vernalization, while plants with recessive *VRN1* alleles flower only after vernalization. In winter wheat, *VRN1* transcription requires vernalization, while in spring wheat *VRN1* transcription is independent of vernalization (Yan *et al*. 2003). In winter wheat, vernalization induces *VRN1* transcription in both apices and leaves, and the gradual increase of *VRN1* mRNA correlates with the gradual effect of vernalization on flowering time, suggesting a direct role of VRN1 in promoting early-flowering (Yan *et al*. 2003). Interestingly, *VRN1* ex-

pression rapidly drops to near pre-vernalization levels after transfer to warm conditions (Yan *et al*. 2003; Fu *et al*. 2006). Thus, the *memory* of vernalization must be located genetically downstream of *VRN1*. In addition to it's role in the vernalization response, *VRN1* has also a more general role for flowering in wheat: The *T. monococcum mvp* (***m**aintained **v**egetative **p**hase*) mutant, which lacks *VRN1*, is unable to switch to flower development indicating that wheat *VRN1* is essential for phase transition in wheat (Shitsukawa *et al*. 2007).

Genetic data show that cereal *VRN2* acts as a dominant flower repressor in non-vernalized plants. Accordingly, the vernalization requirement is determined by allelic variation at *VRN1* and/or *VRN2* in barley and diploid wheat, while vernalization requirement is determined primarily by allelic variation at *VRN1* in polyploid wheat species. Two genes have been identified at the wheat *VRN2 locus – ZCCT1* and *ZCCT2*, both encoding zinc-finger CCT domain proteins with some similarity to CONSTANS (CO) and CONSTANS-like proteins of Arabidopsis (Yan *et al*. 2004). *ZCCT1* is expressed only in non-vernalized plants, and loss-of-function mutations are associated with the *vrn2* early flowering phenotype. Similarly, the barley *ZCCT1* genes are present in 23 winter varieties but completely deleted in 61 spring varieties (Yan *et al*. 2004).

Detailed analyses have shown epistatic interactions between wheat *VRN1* and *VRN2*, suggesting that VRN2 represses *VRN1* (Yan *et al*. 2003, 2004). *VRN1* alleles that confer differential vernalization requirements do not usually vary in the coding region, but three independent deletions were found in the wheat *VRN1* promoter region of spring varieties (Yan *et al*. 2003). These deletions are located near the transcriptional start site, and adjacent to a CArG-box motif (Yan *et al*. 2003). This suggests that a *trans*-acting factor binds to the non-mutated site in the winter varieties and represses *VRN1* prior to vernalization. However, some spring wheat accessions have no mutated promoter sequences, suggesting that additional intragenic regulatory sites exist. Indeed, it was recently reported that a 2.8-kb sequence segment in the first intron of *VRN1* is essential for a vernalization requirement. More than 20 spring wheat accessions have deletions including this 2.8-kb segment (Fu *et al*. 2005). Similarly, dominant *VRN1* alleles for spring growth in barley are often characterized by deletions in the first intron (von Zitzewitz *et al*. 2005).

Despite the epistasis between *VRN2* and *VRN1*, the CArG-box motif adjacent to the sequence variation of the *VRN1* spring alleles suggests that additional MADS-box genes might be involved in the vernalization response of grasses. One such gene is *VRT2*, a member of the StMADS-11 clade that includes Arabidopsis *AGL24* and *SVP* (Kane *et al*. 2005). Both *AGL24* and *SVP* are associated with the transition to flowering and control of meristem fate in Arabidopsis (Hartmann *et al*. 2000; Yu *et al*. 2002). *VRT2* expression follows that of *VRN2*, with high levels present during vegetative development but greatly reduced after vernalization. In yeast-two-hybrid screens, direct interactions between VRT2 and VRN1, VRT2 and VRN2 as well as VRN2 and VRN1 were observed (Kane *et al*. 2005). Moreover, VRT2 was found to bind the *VRN1* promoter *in vitro* and to repress *VRN1* transcription in a tobacco reporter assay (Kane *et al*. 2007). In contrast, VRN2 did not bind *VRN1* sequences but stimulated VRT2's repression of *VRN1*. Thus, it is possible that in the absence of vernalization VRT2 recruits a protein complex containing VRN2, VRT2 and possibly other subunits to the *VRN1* promoter for repression of transcription. Vernalization could repress both *VRT2* and *VRN2* causing activation of *VRN1*. Because increased activity of *VRN1* can repress *VRN2* expression (Trevaskis *et al*. 2006), it is possible that vernalization activates a negative feed-back loop. In barley, Trewaskis and colleagues reported that downregulation of *VRT2* homologs was not necessary for *VRN1* activation (Trevaskis *et al*. 2007b). It was suggested that contrary to the commonly held view, the major effect of vernalization in grasses is to activate *VRN1*, which in turn will repress *VRN2*; in this model, VRN2 represses flowering independently of *VRN1* (Trewaskis *et al*. 2006, 2007a). Future work is needed to clarify the relation of *VRT2*, *VRN2* and *VRN1* in wheat and barley.

In Arabidopsis, the most upstream known regulators of vernalization are VIN3 and the VIN3-like (VIL) proteins (Sung and Amasino 2004; Sung *et al*. 2006b), but it is unknown whether vernalization depends on VIL proteins in other species. Recently, three wheat *VIL* genes (*TmVIL1-3*) were described (Fu *et al*. 2006). Similar to Arabidopsis *VIN3*, *TmVIL1-3* expression is not induced by short exposures to cold, but transcripts accumulate after about 4-6 weeks of cold treatment. In warm conditions, transcript levels rapidly return to pre-vernalization levels. In contrast to Arabidopsis *VIN3*, however, *TmVIL1-3* transcripts are detectable even in the absence of vernalization. Interestingly, *TmVIL1* maps on chromosome 5 close to the vernalization gene *VRN-D5* (Fu *et al*. 2006). It remains to be tested whether VIL proteins do indeed mediate the vernalization response in wheat and other grasses.

Lolium perenne (perennial ryegrass) is a non-cereal grass. QTL mapping suggests that an orthologue of wheat *VRN1* is responsible for differential vernalization requirements (Jensen *et al*. 2005; Andersen *et al*. 2006). In contrast, no ryegrass orthologue of cereal *VRN2* was found so far, although two *VRN2* homologs are located close to a vernalization QTL (Andersen *et al*. 2006). In ryegrass, vernalization was not only studied by QTL mapping but also by transcriptional profiling on cDNA microarrays (Ciannamea *et al*. 2006a). A number of novel putative regulators that respond to vernalization have been identified, including three potential transcriptional regulators: the MADS-box gene *LpMADS1*, the *CONSTANS*-like gene *LpCOL1*, and the *JUMONJI*- (*JmjC*) like gene *LpJMJC* (Ciannamea *et al*. 2006a). *LpMADS1* has been implicated in the ryegrass vernalization response before (Petersen *et al*. 2004; Jensen *et al*. 2005; Andersen *et al*. 2006; Petersen *et al*. 2006). CONSTANS-like genes are involved in the control of flowering by photoperiod in Arabidopsis and rice and by vernalization in wheat and barley. In contrast, JmjC-domain proteins were not yet related to the vernalization response. However, members of this protein family are involved in floral transition in Arabidopsis (Noh *et al*. 2004). In the absence of one of these proteins, RELATIVE OF EARLY FLOWERING 6 (REF6), the promoter region and first intron of *FLC* become hyperacetylated (Noh *et al*. 2004). This led to the suggestion of a role for JmjC-domain proteins in histone deacetylation to repress *FLC*. Although *LpJMJC* is not a close homolog of *REF6*, it may act at the molecular level also through chromatin remodeling. This is supported by the recent finding that many JmjC-domain proteins have histone-demethylase activity (Klose *et al*. 2006). Further work will reveal if LpJMJC proteins do in fact promote epigenetic regulation of vernalization-responsive genes in grasses, paralleling the regulation of *FLC* and *AGL19* in Arabidopsis.

A vernalization gene with dominant spring growth habit has been mapped to the same location in barley, wheat and ryegrass, indicating that *VRN1* is evolutionary-conserved among cereals. This supports a monophyletic origin of the vernalization pathway in cereals, which like other recent temperate grasses evolved from subtropical primitive grasses that probably had no vernalization requirement. The development of a vernalization pathway was an important step in the spread of the grasses to the cold regions. In most wild Triticeae species, vernalization promotes flowering, suggesting that the winter growth habit is the ancestral state in this group of species. Because only eudicots, but not monocots have *FLC* genes for the vernalization pathway, vernalization probably evolved independently in these groups. However, for both, the vernalization pathway involves at least one repressor (*FLC* in Arabidopsis, *VRN2* in grasses) that is downregulated by vernalization and at least one activator (*SOC1*, *AGL24*, *AGL19* in Arabidopsis, *VRN1*

in grasses) that is induced by vernalization.

CROSSTALK BETWEEN VERNALIZATION AND PHOTOPERIOD PATHWAYS

Vernalization occurs in winter when days are short, and therefore it is not surprising that vernalization and photoperiod pathways appear to interact. Wheat, for instance, is originally a SD-LD plant and growth first in short-day photoperiods (SD) and then in long-day photoperiods (LD) can efficiently induce flowering of many winter varieties in the absence of vernalization (McKinney and Sando 1935; Evans 1987). This dual flowering induction requirement is also characteristic of many other winter grasses (Heide 1994). At the molecular level, the effect of vernalization on wheat *VRN2* can be mimicked by a transient exposure to SD followed by return to LD conditions (Dubcovsky *et al*. 2006). Despite the repression of *VRN2* in SD, *VRN1* is not expressed until plants are transferred to LD. Thus, it is likely that *VRN1* expression is not only down-regulated by VRN2 but also involves a LD-dependent activator.

This idea was supported by the finding that wheat and barley *VRN3* encode homologs of FT (Yan *et al.* 2006). *VRN3* is upregulated in LD and is repressed by VRN2. This resembles the situation in rice, where the VRN2 homolog Hd1 represses the *VRN3* homolog *Hd3a*, but not the situation in Arabidopsis, where the VRN2 homolog CO activates the *VRN3* homolog *FT* (Searle and Coupland 2004). In SD, *VRN2* is repressed but *VRN3* levels remain low, and therefore plants will not flower until they are transferred to LD, where elevated *VRN3* levels lead to *VRN1* activation (**Fig. 4**). It is, however, not known how LD activates VRN3 and whether this involves another CO homolog.

Similar to the LD requirement of wheat *VRN1*, the ryegrass orthologue of wheat *VRN1*, *LpMADS1*, is not only induced by vernalization (Ciannamea *et al*. 2006a) but is also one of the first transcribed genes after exposures to LD (Petersen *et al*. 2004). Recently, Trevaskis and colleagues (2006) have studied the regulation of the barley *VRN1* and *VRN2* genes by both vernalization and photoperiod. These authors demonstrated that barley *VRN2* is mainly controlled by photoperiod while barley *VRN1* is mainly controlled by vernalization and developmental status (Trevaskis *et al*. 2006). Based on these data they proposed a model for the vernalization response in cereals, where VRN2 would function primarily in a photoperiod-dependent manner, to prevent flowering in LD when plants have not been vernalized and not exposed to SD. *VRN1* would also be sensitive to day length, but it would act as the primary target of vernalization; during winter, the increase in *VRN1* levels effectively repress *VRN2*, enabling *VRN3* to respond to photoperiod induction, finally leading to the competence to flower (Trevaskis *et al*. 2006, 2007a). Therefore, *VRN2* would serve as a pathway-integrator, between vernalization and photoperiod, much like Arabidopsis *FLC* (Trevaskis *et al*. 2007a).

Interestingly, also in Arabidopsis a link between photoperiod and vernalization pathways was recently discovered (Sung *et al*. 2006b). This link suggests that similar epigenetic mechanisms are involved in both vernalization and photoperiod pathways in Arabidopsis: while VIN3 is needed to repress *FLC* after vernalization (Sung and Amasino 2004), the VIN3-like VIL1 protein is needed to repress the *FLC*-homolog *FLM* in SD (Sung *et al*. 2006b). Similarly to FLC, which delays flowering in the absence of vernalization (Michaels and Amasino 1999; Sheldon *et al*. 1999), FLM delays flowering in the absence of favorable LD conditions (Scortecci *et al*. 2001, 2003). At least in yeast, the VIL1 and VIN3 proteins interact, and both are needed for epigenetic repression of *FLC*. In contrast, only VIL1 is needed for epigenetic repression of *FLM* (Sung *et al*. 2006b). These pathway-interactions might just be the tip of the iceberg, and future work will reveal how cold and photoperiod signals are integrated.

CONCLUSIONS

Studies using diverse species showed that a vernalization-requirement is a common trait among Angiosperms that most likely evolved independently several times. In Arabidopsis, the *FLC*-dependent pathway is best understood, and recent developments suggest that it could be present in many eudicots. The Arabidopsis vernalization pathways, which are *FLC*-, *AGL24*- or *AGL19*-dependent, are activated by a single cold-responsive element, VIN3, and all share downstream targets such as *AP1* and *LFY*. Regulation

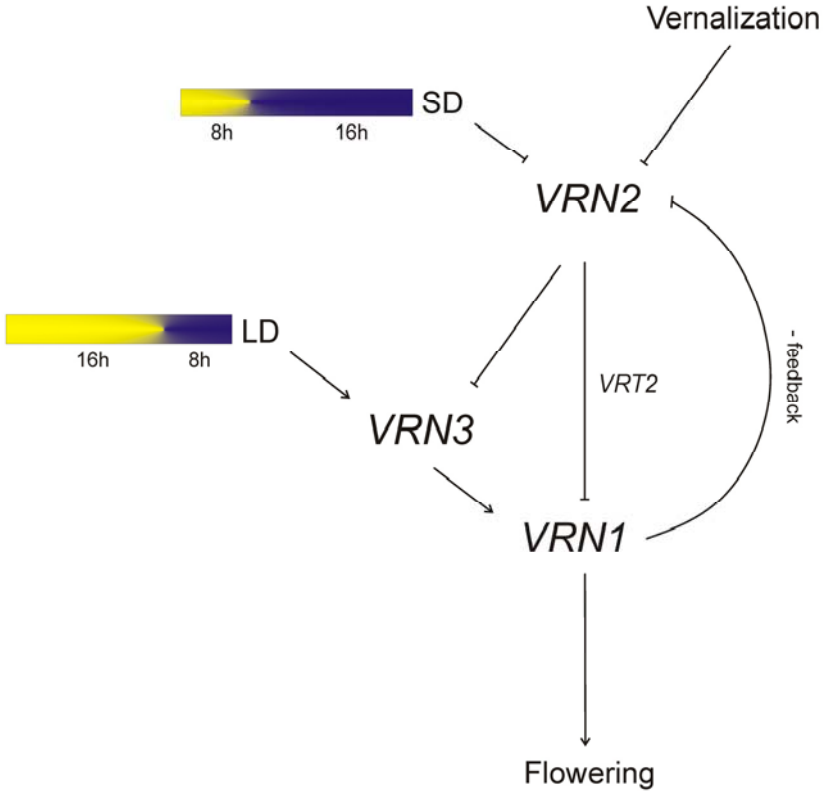

Fig. 4 Crosstalk between vernalization and photoperiod in grasses. Three loci, *VRN1*, *VRN2* and *VRN3*, mediate the vernalization response in grasses. *VRN2* acts to repress *VRN1* and *VRN3*. Repression of *VRN1* involves *VRT2*, and repression of *VRN3* prevents flowering in LD. Vernalization down-regulates *VRN2* and thus lifts repression from *VRN3* and *VRN1*, but full expression of *VRN1* depends on *VRN3* activation by LD. Activation of *VRN1* can also lead to inhibition of *VRN2* via negative feedback. Alternatively, SD can repress *VRN2* and substitute for vernalization. Once *VRN2* levels are low and *VRN3* is activated, *VRN1* can promote transition to flowering.

of the vernalization-specific genes *FLC* and *AGL19* occurs at the epigenetic level, and involves recruitment of histone-modifying PcG complexes, which lead to the establishment of repressed chromatin states (Schönrock *et al*. 2006; Sung and Amasino 2006). This repression is mitotically stable and can persist throughout development. At least two PcG complexes have been proposed to contribute to the vernalization response: the VRN2-complex, which acts as a repressor of *FLC after* vernalization, and the EMF2-complex, which acts as a repressor of *AGL19 before* vernalization. In addition, work in Arabidopsis has established that vernalization has an epigenetic base involving multiple proteins that affect chromatin. It will be of great interest to discover whether such epigenetic mechanisms form the "memory of winter" in other species as well.

ACKNOWLEDGEMENTS

Work in the laboratory of L.H. is supported by SNF project 3100AO-104238, ETH project TH-16/05-2 and a grant from the Zürich-Basel Plant Science Center.

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