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 FLC, 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)GG motif; MADS domain protein binding element; ChIP, chromatin immunoprecipitation; FLC, Flowering Locus C; H3K27me3, 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

**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

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**FLC-Independent Vernalization Responses**

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**ABSTRACT**

The floral activator AGL24

Other species

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CROSSTALK BETWEEN VERNALIZATION AND PHOTOPERIOD PATHWAYS

CONCLUSIONS

ACKNOWLEDGEMENTS

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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 results in 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 Jarovce in Russian (from "Jar": formerly, 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 (Wellsensiek 1962; Wellsensiek 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 niger), 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 direct flowering, not the postulated endogenous 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 action of vernalin cannot be made (Melchers 1939).

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. This treatment was readily made for several winter cereals, but the cold-induced changes must be self-perpetuated throughout plant development, and are closely related to cell proliferation. To this respect, Wellsensiek (1962, 1964) performed a series of experiments with excised portions of leaves of Lunaria bien- nis, 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. Wellsensiek 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 Amaniso 2005; Sung and Amaniso 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 Amaniso 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 SUPPRESSOR 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 Amaniso 1999; Sheldon et al. 1999). In addition to FRI, several other FLC regulators have been identified. Some of them are FRI-dependent, like the VERNALIZATION INDEPENDENT (VIF) genes, which encode subunits of the Pafl-complex. This complex establishes histone H3 lysine 4 trimethylation (H3K4me3) of FLC chromatin to allow efficient transcription (He et al. 2004; Oh et al. 2004). Another activator of FLC, PHOTO- PERIOD INDEPENDENT EARLY FLOWERING1 (PIE1), is similar to ATP-dependent chromatin-remodeling proteins of the ISWI and SWI2/SNF2 family (Noh and Amaniso 2003). Thermo-regulating SWI2/SNF 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 APE- TALAI (API) and LEAFY (LFY) (Fig. 1) (for review see Parey 2005). The mechanism by which FLC represses the apical meristem has been studied in detail: the SOCI promoter region harboring a CArG-box motif (MADS domain protein binding element), since mutations in this CArG-box reduced SOCI repression in transgenic 35S::FLC plants (Hepworth et al. 2002). Chip assays have recently revealed that a direct FLC-SOCI interaction occurs in vivo, and might involve the action of a multi-protein complex (Hellwell et al. 2006; Searle et al. 2006). Notably, the repression of FT expression by FLC may be spatially confined to the flower and 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.
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 H3K4me3 (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; Kondor 2001). Arabidopsis, Drosophila and human, VRN2 and Su(Z)12 are subunits of the Polycar Repressive Complex 2 (PRC2) (Czermak 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 H3K27me3 modifications into the chromatin of target genes; such H3K27me3 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 H3K27me2 and H3K9me2, 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 H3K27me2 seems mostly normal but is not sufficient to maintain FLC repression, suggesting that VRN1 acts downstream of VRN2-mediated H3K27me2 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 H3K9me3 and H3K27me3 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 H3K9me2/3 and H3K27me3 (Turck et al. 2007). During vernalization, LHP1 associates with FLC, where it remains after return to warmer conditions. In vernalized lhp1 mutants, both H3K9me2 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 H3K27me3 marks into FLC chromatin, which in turn help to recruit LHP1 and also VRN1; LHP1 and VRN1 then assist to establish H3K9me2 marks and together are required to maintain FLC transcription shut off.
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.
### 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, aegl19 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 **API** (Schönrock et al. 2006). Over-expression of AGL19 in wild-type plants did not affect **SOC1** expression, and over-expression of AGL19 in a socl 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 aegl19 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 VNR2-complex, AGL19 is repressed before vernalization by the EMF2-complex (Schönrock et al. 2006). This PRC2 complex contains the VNR2 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).

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

<table>
<thead>
<tr>
<th>Species/Loci</th>
<th>Encoded protein (Arabidopsis homologs)</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>AGL19, AGL24</td>
<td>MADS-domain protein (SOC1), MADS-domain protein (SVP)</td>
<td>Activator of flowering</td>
</tr>
<tr>
<td>Cereals</td>
<td>VRN1, VRN2, ZCCT1, ZCCT2, VRN3, VRT2</td>
<td>MADS-domain protein (AP1), CCT domain protein (CO), MADS-domain protein (SVP, AGL24)</td>
<td>Activator of flowering, Repressor of flowering, Putative repressor of flowering</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>LpMADS1, LpMADS10, LpCOL1, LpJMJC</td>
<td>MADS-domain protein (API), MADS-domain protein (SVP), CCT-domain protein (CO), JUMONJI-like protein (ELF6, REF6)</td>
<td>Putative activator of flowering, Putative repressor of flowering, Putative chromatin remodeling activity</td>
</tr>
</tbody>
</table>

Fig. 3 AGL19 and FLC are regulated by distinct PRC2-like complexes. Persistent repression of FLC after vernalization requires the VNR2-complex, which is similar to the metazoan Polycomb Group Repressive complex 2 (PRC2). In addition to VNR2, the VNR2-complex includes FIE and CLF/FSN; 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/FSN) 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 msil-1 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 msil-1 cs plants compared to the wild type (Yu et al. 2002). In contrast to FLC, there was no H3K9met2 detected at AGL19. Because much of the H3K27met3 at AGL19 chromatin disappeared after vernalization, it was concluded that the ERF2-complex, which contains MS11 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 H3K27met3 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 support a model where vernalization in Arabidopsis involves the coordinated action of two independent PRC2-like complexes: the VRN2-complex, which represses FLC, and the ERF2-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 shoot is initiated, the AGL24 transcript is 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 API1 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 SYP (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 api1 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-like 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 (API) (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-
pressure 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 its role in the vernalization response, VRN1 also has a more general role for flowering in wheat: The T. monococcum mvp (maintained vegetative phase) mutant, which lacks VRN1, is unable to switch to flower development indicating that wheat VRN1 is essential for phase transition in wheat (Shitsuikawa 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 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 accessions (Andersen et al. 2005). 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. Therefore it is likely that at least two MADS, all MADS, 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 VRT1 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 (Treviskis et al. 2006), it is possible that vernalization activates a negative feed-back loop. In barley, Treviskis and colleagues reported that downregulation of VRT2 homologs was not necessary for VRN1 activation (Treviskis 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 (Treviskis 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 VRN3 and the VRN3-like (VIL) proteins (Sung and Amasino 2004; Sung et al. 2006b), but it is unknown whether vernalization mediates their flowering in other species. Recently, three wheat FIL genes (TmVIL1-3) were described (Fu et al. 2006). Similar to Arabidopsis VRN3, 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 VRN3, 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 requirement in barley (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 (Ciannaee 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 (Ciannaee 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 close homoeolog of the S. C. F6, part of the S. C. F6 may play a role 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, parallelizing the regulation of FLC and AGL19 in Arabidopsis.

The 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 towards the cold regions. In most wild Triticeae specios, 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...
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 photoperiods (SD) and then in long-day photoperiods (LD) appears to interact. Wheat, for instance, is originally a SD-LD plant and growth first in short-day photoperiods. Vernalization occurs in winter when days are short, and 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 rye-grass orthologue of wheat VRN1, LpMADS1, is not only induced by vernalization (Ciannaméa 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 demonstrate 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 (Scortecchi 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 expression and vernalization, and the Ezh2-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.

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