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Regulation of flowering in temperate cereals

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Long exposure to cold (vernalization) accelerates flowering in winter cereals, a process regulated by the *VRN1* (≈*AP1*), *VRN2*, and *VRN3* (≈*FT*) vernalization genes. Flowering during the fall is prevented by the *VRN2* downregulation of *VRN3* and low *VRN1* transcription. Vernalization induces *VRN1*, which is followed by the downregulation of *VRN2*, thereby releasing *VRN3*. In the longer days of spring, photoperiod genes *PPD1* and *CO* upregulate *VRN3*, which induces *VRN1* above the threshold levels required for flowering initiation. *VRN3* transcription is modulated through interactions involving CCT-domain proteins and HAP2/HAP3/HAP5 complexes coded by multiple genes. The vast number of HAP–CCT combinations can provide the flexibility required for integrating seasonal cues and different stress signals in the regulation of the transition to flowering.

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Introduction

The adaptability of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) to a wide range of environments has been favored by allelic diversity in genes regulating growth habit (*VRN* genes) and photoperiod response (*PPD* genes) [1]. Differences in the *PPD* genes divide the temperate cereals into photoperiod-sensitive and photoperiod-insensitive classes, whereas differences in the *VRN* genes divide them into winter and spring classes. Winter cereals are planted in fall and require long exposures to cold temperatures to accelerate flowering (vernalization requirement), an adaptation that prevents the exposure of sensitive floral meristems to freezing winter temperatures. Spring cereals are usually planted in spring and do not have a vernalization requirement.

Natural variation in vernalization requirement in the temperate cereals is mainly associated with allelic differences in the *VRN1*, *VRN2*, and *VRN3* vernalization genes. ***VRN1*** encodes a MADS-box transcription factor with high similarity to *Arabidopsis* meristem identity genes *APETALA1*, *CAULIFLOWER*, and *FRUITFULL* [2••,3,4], which regulate the transition of the vegetative shoot apical meristem to the reproductive phase [5]. In diploid wheat, radiation-induced deletions including the *VRN1* gene result in plants that never flower, suggesting that this gene is essential for flowering in this species [6•]. The ***VRN2*** region includes two similar *ZCCT* genes encoding proteins with a putative zinc finger and a CCT domain that have no clear homologs in *Arabidopsis* [7•]. These two *ZCCT* genes act as flowering repressors, and nonfunctional mutations or simultaneous deletions of both genes are associated with spring growth habit in wheat [7•,8] and barley [9,10]. ***VRN3*** encodes a RAF kinase inhibitor like protein with high homology to *Arabidopsis* protein FLOWERING LOCUS T (FT) [11•]. FT acts as a long-distance flowering signal (florigen) that moves from leaves to apices [12,13•] and promotes flowering in a diversity of plant species by inducing meristem identity genes [14]. A similar mechanism has been recently demonstrated in wheat [15].

Natural variation in the response to photoperiod is mainly determined by allelic differences in the *PPD1* gene, a member of the pseudoresponse regulator (PRR) gene family [16•]. In photoperiod-sensitive wheat and barley varieties, *PPD1* accelerates flowering by upregulating *VRN3* under long day conditions. In barley, a nonfunctional mutation in the CCT domain of *PPD1* alters the circadian timing of *CONSTANS* (*CO*) and limits the induction of *VRN3* by long days [16•] (Figure 1), resulting in a late flowering recessive *ppd1* allele. In wheat, a 2 kb deletion in the *PPD-D1* promoter is responsible for a semidominant photoperiod-insensitive allele for early flowering associated with misexpression of *PPD-D1* and increased expression of *VRN3* under short days [17].

The role of *VRN3* as an integrator of the vernalization and photoperiod pathways in temperate cereals is well documented [11•,18]. However, the integration of many other environmental cues that affect flowering time (e.g. nitrogen availability, stress conditions, temperature fluctuations, etc.) is just starting to be unraveled. This review summarizes our current knowledge of the gene network responsible for the integration of these multiple signals into the regulation of flowering initiation in wheat and barley.

A model integrating vernalization and photoperiod pathways

Figure 1 presents a schematic model illustrating the expression of the main vernalization and photoperiod

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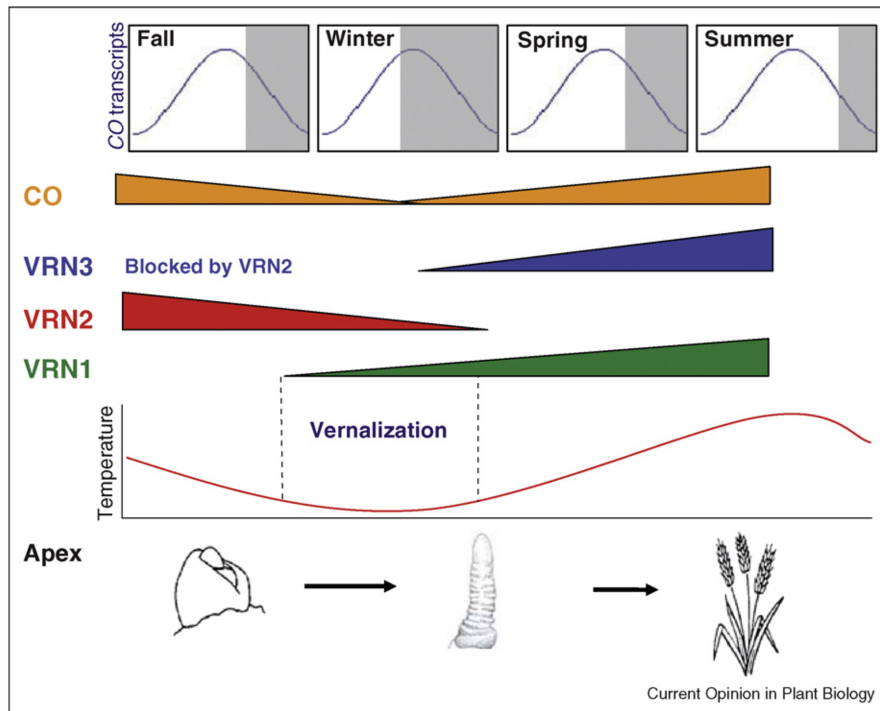
Figure 1



Regulation of vernalization and photoperiod genes by environmental changes during the growing season in a photoperiod-sensitive winter cereal. The red line at the bottom represents the seasonal variation in temperature and the four rectangles on top indicate the duration of light (white) and dark (shaded) periods during typical days of the different seasons. The curves in blue represent the variation of *CONSTANS* (*CO*) transcript levels in the leaves during the day. According to the external coincidence model the post-translational activation of CO occurs only when *CO* mRNA is expressed in the light, which occurs only under long days (for a recent review see [14]). Higher levels of CO protein (represented in orange) favor the activation of *VRN3* (blue) during long days only when the *VRN2* (red) repressor is not present in the leaves. *VRN1* (green) is slightly upregulated during winter and then strongly induced by *VRN3* in the apices when daylength increases. When *VRN1* reaches a threshold level, the vegetative apex (left) stops producing leaves and starts producing spikelet meristems (double-ridge state, center). Most temperate cereals require long days to complete the subsequent spike development and elongation phases (right).

genes during the growing season in a photoperiod-sensitive winter cereal (ancestral type). After germination in fall, when days are still long, *VRN3* is repressed by high levels of *VRN2*, precluding the induction of *VRN1*. *VRN1*, initially transcribed at very low levels in leaves and apices, is gradually upregulated during the short, cold days of winter, and downregulates *VRN2*. The low levels of *VRN2* transcripts in the leaves facilitate the upregulation of *VRN3* by long days in the spring, a process mediated by photoperiod genes *PPD1* and *CO* [16•]. VRN3 is exported to the shoot apex where it further promotes *VRN1* transcription above the threshold levels required for the induction of flowering. The experimental evidence supporting the gene interactions presented in this model is discussed below.

VRN3 promotes *VRN1* transcription under long days

In *Arabidopsis*, a protein complex including FT (the VRN3 homolog) and the bZIP protein FD binds to the promoter of *APETALA1* and *FRUITFUL* (the *VRN1* homologs). The upregulation of these meristem identity genes accelerates the transition between the vegetative and reproductive apices [19]. Similar interactions between VRN3 and the FD-LIKE2 (FDL2) proteins and between FDL2 and the *VRN1* promoter have been observed in wheat, suggesting a conserved regulatory mechanism [15] (Figure 2a).

In wheat, the insertion of a transposable element in the promoter of a *VRN3* allele is associated with higher *VRN3* transcript levels [11•]. Transformation of winter wheat plants with this *VRN3* allele results in parallel increases in *VRN1* transcript levels and early flowering (Figure 2b) [15]. Similarly, introgression of a *VRN3* allele for early flowering into winter barley varieties upregulates *VRN1* and eliminates the vernalization requirement [11•]. These results confirm the ability of *VRN3* to upregulate *VRN1*.

VRN2 represses *VRN3* under long days

The role of *VRN2* in the direct or indirect repression of *VRN3* is supported by lower *VRN3* transcript levels in diploid wheat lines with functional *VRN2* alleles relative

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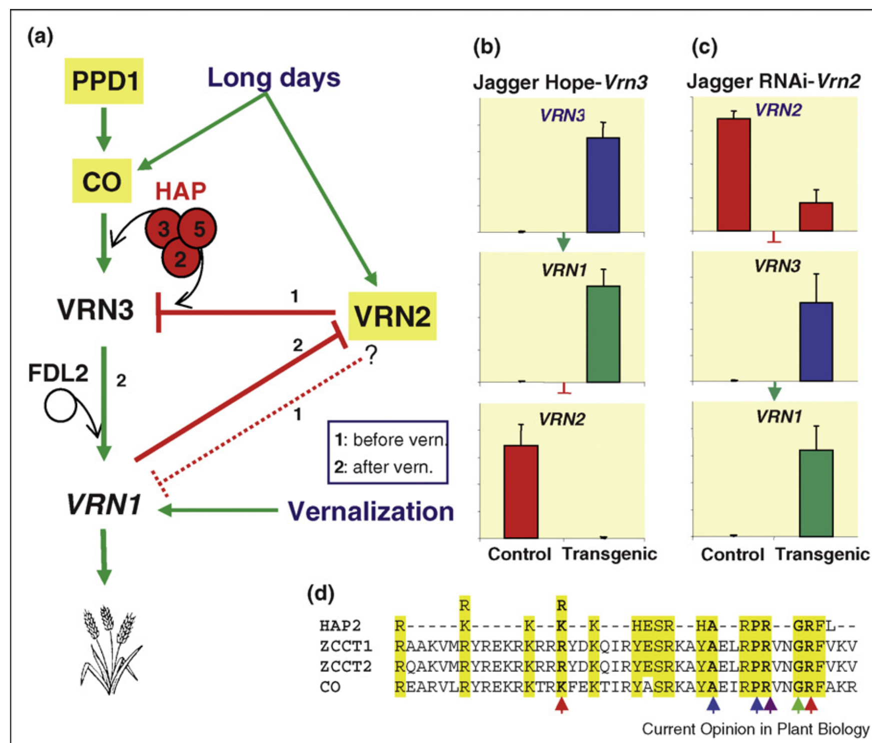
Figure 2



(a) Combinatorial model of flowering. The *VRN3* gene integrates photoperiod (*PPD1–CO*) and vernalization (*VRN1–VRN2*) with other environmental signals (e.g. stress) through interactions between CCT-domain proteins (yellow highlight) and trimeric HAP2/3/5 complexes (red circles). Interactions between VRN3 and FDL2 then upregulate *VRN1* overcoming its initial downregulated state. Vernalization induces *VRN1* under both short and long days, which then represses *VRN2*. Dotted lines indicate potential interactions that require additional experimental validation (see text). The interactions among the three vernalization genes generate a feedback regulatory loop that once started, leads to an irreversible induction of flowering. The proposed interactions in the model are supported by experiments in which the winter wheat variety Jagger was transformed with **(b)** the dominant *Vrn3* allele from Hope [11•] or with **(c)** an RNAi construct for *VRN2* [7•]. Under long days, the upregulation of *VRN3* transcripts in leaves of transgenic plants results in the upregulation of *VRN1* [11•] and the downregulation of *VRN2* (not reported before), whereas RNAi downregulation of *VRN2* results in the upregulation of *VRN3* in the leaves (not reported before) and *VRN1* in both leaves and apices [7•]. (d) Conserved amino acids between CCT domains from ZCCT and CO proteins and HAP2 subunits. Arrows indicate the location of nonfunctional mutations in PPD1 (green), CO (blue), ZCCT (red), or both CO and ZCCT (violet) [8].

to isogenic lines with nonfunctional *VRN2* alleles [11•] and by the downregulation of *VRN3* in transgenic barley plants overexpressing *VRN2* relative to nontransgenic controls [20•]. The reciprocal result is observed in transgenic RNAi::VRN2 wheat plants, in which reduced *VRN2* transcript levels [7•] are associated with significantly higher *VRN3* transcript levels ($P < 0.0001$, Figure 2c). An independent, but indirect argument supporting the role of *VRN2* as a long-day repressor of *VRN3* is the recent discovery of similar interactions between their closest rice homologs, *GHD7* (*VRN2* homolog) and *HD3a* (*VRN3* homolog). *GHD7* is a long-day repressor of flowering in rice, and enhanced expression of *GHD7* downregulates *HD3a* under long days but not under short days [21].

Finally, the hypothesis that the *VRN2* repression of flowering is mainly mediated by its effect on *VRN3* (Figure 2a) is supported by the late flowering phenotype of photoperiod-insensitive (*ppd1*) barley lines lacking the *VRN2* genes [20•]. In *ppd1* barley lines, *VRN3* is not efficiently induced by long days and therefore, the *VRN2* deletions can not confer the earlier flowering phenotype observed in the photoperiod-sensitive lines.

VRN1 represses *VRN2* transcription

Mutations in the regulatory regions of *VRN1* result in dominant *Vrn1* alleles, which are expressed with or without vernalization conferring a spring growth habit. In isogenic lines of hexaploid wheat carrying different combinations of dominant and recessive *VRN-A1*, *VRN-B1*, and *VRN-D1* alleles, only transcripts of the dominant alleles are detected in leaves of unvernalized plants at the first-leaf stage to third-leaf stage grown under long days; but both dominant and recessive *VRN1* alleles become abundant a few weeks later (sixth-leaf stage) [22]. A reduction in the transcript levels of *VRN2* is observed after the transcription of the dominant *Vrn1* allele and before the transcription of the recessive alleles,

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a phenomenon that is not observed in isogenic control plants with all three recessive *vrn1* alleles. This suggests that the dominant *Vrn1* allele (or a gene regulated by *VRN1*) downregulates the transcription of *VRN2* [22]. This interaction is also supported by the observed reduction of *VRN2* transcript levels ($P < 0.0001$) in winter wheat lines transformed with the dominant *Vrn3* allele (Figure 2b), which exhibit higher *VRN3* and *VRN1* transcript levels than nontransgenic controls [15].

VRN1 is likely the primary target of the vernalization signal

The interactions among *VRN1*, *VRN2*, and *VRN3* genes form a feedback regulatory loop and, therefore, modification of the transcript levels of any one of these genes affects the transcript levels of the others, complicating the determination of the primary target of vernalization. Experiments under short days are helpful to separate some of these effects as *VRN3* and *VRN2* transcript levels are very low under this condition [23,24,25•]. Under short days *VRN1* transcripts are significantly higher in vernalized than in unvernalized plants [24,25•,26] suggesting that *VRN1* is upregulated by vernalization independently of the other two genes [18,25•]. According to this model, the observed downregulation of *VRN2* (in apices and leaves) and upregulation of *VRN3* (in leaves) during vernalization are the indirect effect of the upregulation of *VRN1* (Figure 2a) [18].

VRN1 transcript levels in unvernalized plants grown under short days are low, but still significantly higher than in unvernalized plants grown under long days. This difference can be explained by the short day repression of *VRN2*, assuming that *VRN2* can repress *VRN1* independently of its effect on *VRN3* (Figure 2, dotted line); or by the short day downregulation of a different *VRN1* repressor. Additional experiments are necessary to distinguish these two alternative hypotheses.

VRN1 has multiple regulatory regions

Since *VRN1* is probably the primary target of vernalization [25•] and is essential for flowering [6•], the characterization of the regulatory regions identified in the promoter and first intron is critical to understand the regulation of flowering initiation in the temperate cereals.

The importance of the *VRN1* first intron in maintaining low levels of *VRN1* transcripts before vernalization is supported by the discovery of at least 15 independent deletions and 2 repetitive elements insertions in this region associated with high levels of *VRN1* transcripts and a spring growth habit in both wheat and barley [10,24,27•,28,29]. These indels may eliminate a binding site for a putative repressor that is downregulated by vernalization [27•] or affect a site required to establish a repressed state of the intron one chromatin [18]. Interestingly, transcript levels of barley *Vrn1* alleles with large deletions in the first intron still show a twofold to threefold increase after vernalization under short days [29], suggesting that regions outside the first intron can also regulate the *VRN1* response to vernalization. This agrees with the observation that deletions in some regions of the *Vrn1* promoter can reduce or eliminate the vernalization requirement [2••,24,30,31].

A CArG-box (MADS-box binding site) in the *VRN1* promoter (Figure 3) was initially proposed as a critical regulatory site for vernalization [2••]. However, the discovery of a diploid wheat accession with a complete deletion of the CArG-box and a winter growth habit suggests that the vernalization regulatory site is located upstream of the CArG-box (putative VRN-box, Figure 3) [30,31]. The MADS-box transcription factor VRT2 binds *in vitro* to the *VRN1* promoter in the CArG-box region [32,33] (Figure 3) and represses *VRN1* in a tobacco reporter assay (*VRN1*-promoter::GFP) [34]. The VRT2 protein also interacts with VRN2, and when both are transformed together in tobacco they show stronger repression of the *VRN1*-promoter::GFP reporter [34]. However, it is unlikely that *VRT2* acts as a vernalization repressor [32,34] since the CArG-box region is not a critical region for the regulation of vernalization (Figure 3) [31]. Additional research would be necessary to determine if *VRT2* is involved in the regulation of flowering initiation or if it regulates *VRN1* transcription later, during flower meristem identity determination [33,35].

The CArG-box seems to be involved in photoperiod regulation. In diploid wheat, alleles with indels affecting both the CArG-box and the VRN-box (*Vrn1g*, Figure 3) or the CArG-box and first intron (*Vrn1f*, Figure 3) are associated with significantly higher *VRN1* transcripts under short days than other alleles [24]. This suggests that the CArG-box may be the target of an unknown *VRN1* repressor that is active under short days [24]. Lines carrying the *Vrn1f* or *Vrn1g* alleles show an earlier transition of the shoot apices to the double-ridge stage under short days relative to other alleles, but they still require long days for a timely completion of later phases including spike development and elongation. This observation suggests that there are additional regulatory checkpoints after the induction of *VRN1* and the transition to the double-ridge stage [24].

Upstream of the CArG-box and VRN-box, four ACGT-core binding sites have been shown to be the targets (at least *in vitro*) of FDL2 (Figure 3). Since FDL2 interacts with VRN3 [15], these are likely the regulatory sites involved in the upregulation of *VRN1* by *VRN3* under long days (Figure 2a).

The CCT–HAP combinatorial model of flowering regulation

The CCT domains share several similar amino acids with the yeast HEME ACTIVATOR PROTEIN2 (HAP2), a

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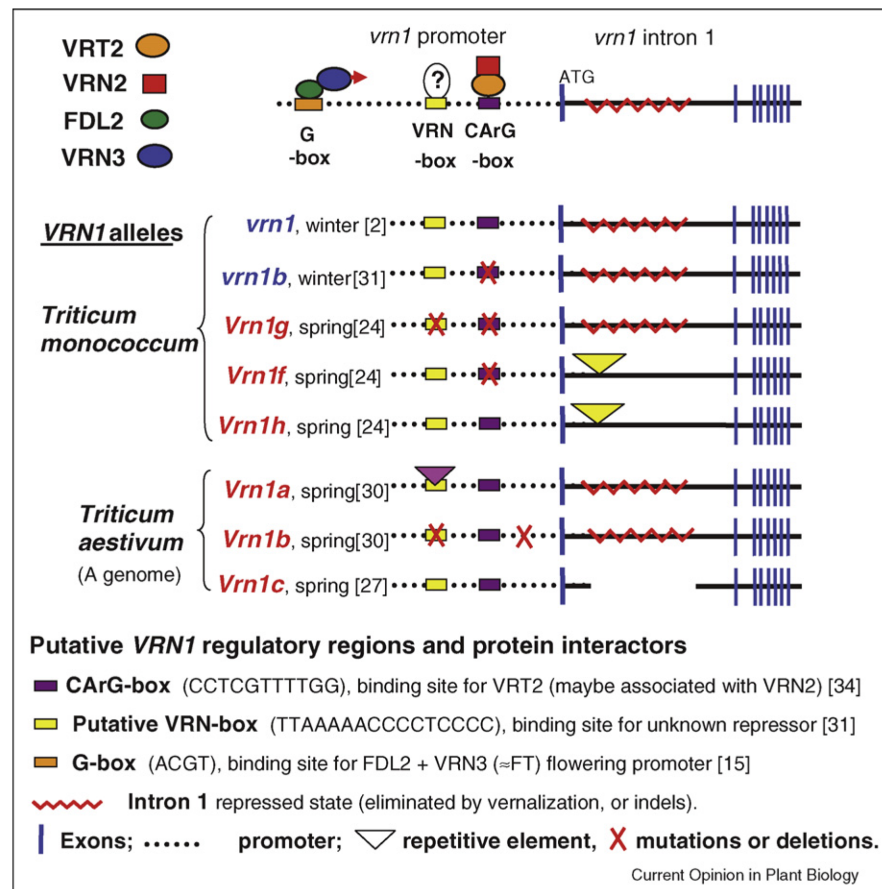
Figure 3



Putative *VRN1* regulatory regions and their variation in different spring alleles (*Vrn1*) in diploid and hexaploid wheat. The mutated or deleted domains in the different alleles are marked with a red X, and the insertions of repetitive elements by inverted triangles of different colors. The wild-type recessive *vrn1* allele, characteristic of diploid and polyploid accessions with a winter growth habit, is listed on top. See text for a detailed explanation.

subunit of the HAP2/HAP3/HAP5 complex that binds to CCAAT-box *cis*-regulatory elements in the promoters of many eukaryotic genes [36,37••,38] (Figure 2d). Mutations at six of these conserved positions in the CCT domains of ZCCT1 and ZCCT2 [7•,8], PPD1 [16•], and CO [39] (Figure 2d) are associated with differences in flowering time. These conserved positions are located in a HAP2 subdomain involved in the interactions with HAP3 and HAP5, and the recognition of the CCAAT-box.

On the basis of the interactions observed between the CCT domains of CO and CONSTANS-LIKE (COL) proteins and the HAP3 and HAP5 subunits in *Arabidopsis*, it has been suggested that the transcriptional regulation of the CCT targets might be mediated by their interactions with different HAP2/3/5 complexes [36,37••]. Overexpression of *Arabidopsis HAP3b* was shown to promote early flowering probably through an interaction with CO or COL proteins, whereas the null mutant *hap3b* delayed flowering under long days but not under short days [40]. In contrast, overexpression of *Arabidopsis HAP2a* or *HAP3a* in phloem companion cells delayed flowering and significantly reduced *FT* transcript levels without affecting *CO* transcript levels [37••]. The delay in flowering was observed only under long days and was counterbalanced by *CO* overexpression, suggesting that CO-dependent activation of *FT* was impaired. Wenkel *et al.* [37••] proposed that CCT-proteins act by replacing the HAP2 subunit of the HAP2/3/5 complex, altering the ability of this complex to bind to the CCAAT-boxes in the promoters of target genes (Figure 2).

The observation that mutations resulting in nonfunctional ZCCT alleles are all located in amino acids conserved between CCT and HAP2 domains [8], together with our unpublished results which show the existence of direct interactions between ZCCT and wheat HAP proteins in yeast two-hybrid systems (C Li, A Distelfeld and J Dubcovsky, unpublished), suggests that the VRN2 repression of *VRN3* (=*FT*) transcription may be mediated by similar interactions with the HAP complex as those described for CO in *Arabidopsis* (Figure 2).

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Each of the HAP subunits is encoded by single genes in yeast and mammals but by multiple genes (≈10) in plants [41–43]. The expansion of the HAP gene families in plants is even larger if the related CCT domains are included (Figure 2d). Together, the multiple CCT-domain proteins able to interact with the HAP2/3/5 complex and the multiple genes that code for each of the HAP subunits have the potential to generate a vast number of molecular combinations. Such putative CCT–HAP interactions provide a flexible system to accommodate the many developmental and environmental signals (e.g. photoperiod, vernalization, stress, etc.) that are integrated in the transition to flowering. For example, *Arabidopsis HAP3a* and *HAP3b* transcript levels are regulated by drought [44] and osmotic stress [45], respectively, and the alteration of their transcript levels results in differences in flowering time. According to this model, the competition among different CCT and HAP proteins can generate complexes of different promoting strengths or binding specificities that can alter *VRN3* transcript levels and, thus, *VRN1* levels and flowering time.

Conclusions and future directions

The initiation of flowering depends on a delicate balance between repressing and promoting forces operating on *VRN1* regulatory regions. *VRN3* plays a central role in this balance by integrating the competing forces of *VRN2* (repression regulated by vernalization) and *PPD1–CO* (promotion regulated by photoperiod). The model presented here suggests that additional environmental signals can be integrated in the regulation of *VRN3* through the interactions between HAP2/3/5 complexes and CCT-domain proteins such as CO and VRN2. The balance among competing CCT-proteins and HAP subunits can affect the transcript levels of *VRN3* and the timing of flowering initiation. The HAP–CCT combinatorial model of flowering has the potential to expand our understanding of the regulation of flowering initiation beyond the vernalization and photoperiod signals. However, this will require a more precise characterization of the different HAP subunits and their interactions with the different CCT-proteins in the temperate cereals.

Additional research will be also necessary to clarify the mechanisms responsible for the upregulation of *VRN1* by vernalization and to identify the genes that regulate *VRN1* independently of *VRN3* and the ones that affect heading time after the expression of *VRN1* and the transition to the double-ridge state [24].

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