

# Genes and gene networks regulating wheat development

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## ABSTRACT

Plants exhibit a complex regulation of the transition of the shoot apical meristems from vegetative to reproductive stages to optimize flowering time and seed production. We positionally cloned three genes showing natural variation in the regulation of wheat flowering in response to vernalization (the exposure to cold temperatures for an extended period of time). The most critical one is *Vrn-1* (a homologue of Arabidopsis *API*) since its absence results in plants that never flower. *Vrn-1* is regulated by *Vrn-2* (a CCT transcription factor, absent in Arabidopsis) that acts as a flowering repressor and by *Vrn-3* (a homologue of Arabidopsis *FT*, designated hereafter *TaFT*), which promotes flowering. VRN-3 interacts with the bZIP protein FDL2, which binds *in vitro* to five elements (core sequence ACGT) present in the *Vrn-1* promoter. A putative CARG box, a binding site for MADS-box genes, is present downstream of the ACGT elements. Genetic studies in *T. monococcum* using a *Vrn-1* allele with a CARG box deletion confirmed that this regulatory element is not essential for the vernalization-mediated repression of *Vrn-1*. However, deletions affecting this regulatory element were associated with increased *Vrn-1* transcription under short days. Electrophoresis Mobility Shift Assays (EMSA) experiments confirmed that this CARG box is the binding site for *TaVRT-2*, a MADS-box gene upregulated by vernalization. Additional *Vrn-1* regulatory sites are located within the first intron. Epistatic interactions between *Vrn-2* and *Vrn-1* using different *Vrn-1* alleles suggest that the regulatory region within the *Vrn-1* first intron has a stronger effect than the one upstream of the CARG box in the promoter in eliminating the effect of *Vrn-2* allelic variation on flowering time. EMSA and Chromatin Immunoprecipitation (ChIP) experiments failed to show a direct interaction between VRN-2 and *Vrn-1* regulatory regions, suggesting an indirect interaction.

The central role of *Vrn-2* in the determination of winter growth habit in tetraploid wheat was confirmed by generating a double recessive *vrn-A2 vrn-B2* line. This line showed a spring growth habit in spite of the presence of homozygous *vrn-1* alleles for winter growth habit. This double recessive line was used to discover functional and non-functional alleles of *Vrn-A2* and *Vrn-B2* in tetraploid wheat.

## INTRODUCTION

The transition between the vegetative and reproductive apices in Arabidopsis is controlled mainly by the meristem identity gene *API*, but is also affected by the paralogous MADS-box genes *FUL* and *CAL*.

Arabidopsis triple mutants for these genes fail to flower<sup>1</sup>. The duplications that originated these three paralogous genes in Arabidopsis occurred after the divergence between dicots and monocots. In the temperate cereals the functional homologue of these three genes seems to be the single copy vernalization gene *Vrn-1*<sup>2</sup>. Deletions of *Vrn-1* in diploid wheat are sufficient to prevent flowering completely under any environmental condition<sup>3</sup> suggesting that there are no functionally redundant paralogues of this gene in the temperate cereals as in Arabidopsis.

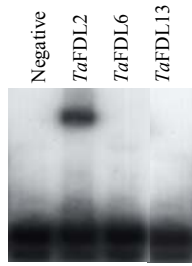
Since *Vrn-1* expression is required for the initiation of the reproductive phase, the characterization of its regulatory regions and interacting genes is central to our understanding of the regulation of flowering initiation in temperate cereals. A large proportion of the natural variation in vernalization requirement in the *Triticeae* species is associated with polymorphisms in regulatory regions in the *Vrn-1* promoter<sup>2,4,5</sup> and its first intron<sup>6,7</sup>. In diploid *Triticeae* species such as *T. monococcum* and barley, natural allelic variation in vernalization requirement is also associated with the *Vrn-2* locus<sup>8</sup>. In these two diploid species *Vrn-2* deletions or mutations in its coding region are associated with a recessive spring growth habit<sup>8</sup>. We discuss here the effect of different *Vrn-1* and *Vrn-2* natural allelic variants on flowering time in wheat and compare it with barley. We also present a model summarizing our current knowledge of flowering regulation in wheat.

## RESULTS

**Interactions between *TaFT* and *TaFDL2*, and between *TaFDL2* and the *Vrn-1* promoter.** Using yeast-2-hybrid assays we found that *TaFT* (VRN-3), which integrates photoperiod and vernalization signals, interacts with bZIP proteins *TaFDL2* and *TaFDL6*. We also showed that *TaFDL2* can interact *in vitro* with five ACGT elements in the promoter of the meristem identity gene *Vrn-1* (Fig. 1), suggesting that *TaFDL2* is a functional homologue of Arabidopsis *FD*<sup>9</sup>. No direct interactions between the *TaFT* protein and the *Vrn-1* promoter were detected.

Transgenic wheat plants overexpressing *TaFT* showed parallel increases in *Vrn-1* transcripts, suggesting that *TaFT* provides transcriptional activation to *Vrn-1*, possibly through interactions with the *TaFDL2* protein. High levels of *TaFDL2* transcripts were observed in the wheat leaves suggesting that *TaFT* is the limiting factor for the activation of *Vrn-1*. This was supported by the fast induction of *Vrn-1* transcripts in transgenic winter

wheat lines over-expressing *TaFT*<sup>9</sup>. In Arabidopsis, *API* is expressed at high levels in the leaves only when *FD* is ectopically expressed in transgenic 35S::*FD* plants.



**Fig 1.** *TaFIDL2* protein binds to *Vrn-1* promoter (EMSA). G-box bZIP binding site (cACGTg) from the *Vrn-1* promoter labelled and used as probes in binding reactions with recombinant proteins *TaFIDL2*, *TaFIDL6*, and *TaFIDL13*.

**The CARg box regulatory site in the *Vrn-1* promoter is not necessary for vernalization requirement.** Based on natural allelic variation in the *Vrn-1* promoter in diploid wheat *T. monococcum* we suggested that the CARg box located upstream of the transcription initiation site might be an important regulatory element for vernalization<sup>2</sup>. To test this hypothesis we produced two segregating populations using *T. monococcum* accession PI355515 as a parent. This accession has a 48-bp deletion in the *Vrn-1* promoter which includes the CARg, and a recessive *vrn-2* allele, which is known to confer a spring growth habit.

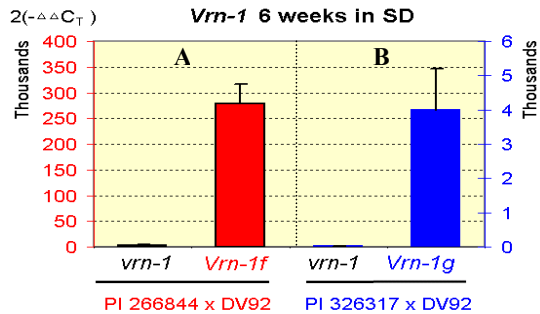
In the F<sub>2</sub> population from the cross PI355515 with winter line G3116 (*vrn-1 Vrnl-2*) all the lines carrying the dominant *Vrn-2* allele showed a strong winter growth habit regardless of the *Vrn-1* allele, whereas all the plants carrying the recessive *vrn-2* allele showed a spring growth habit. This result suggested that the 48-bp deletion was not affecting the vernalization requirement.

We confirmed this result in a segregating population from the cross between PI355515 and spring line PI266844. PI266844 has a dominant *Vrn-2* allele (winter) and a dominant *Vrn-1lf* allele (spring), which has a 1bp deletion in the CARg box and a repetitive element inserted in the first intron<sup>5</sup>. Lines homozygous for the recessive *vrn-2* allele or carrying at least one copy of *Vrn-1lf* showed a spring growth habit, whereas lines homozygous for the allele with the 48 bp deletion and carrying at least one functional copy of the *Vrn-2* repressor flowered approximately 60 days later. Based on these results we concluded that the allele with the 48-bp deletion is a recessive *vrn-1* allele, which confers a vernalization requirement as strong as the one from winter accession G3116 (in the presence of *Vrn-2*). We also concluded that the CARg box located within this 48-bp deletion is not required for the vernalization regulated repression of *Vrn-1*.

**The CARg box is likely involved in the SD repression of *Vrn-1*.** In *T. monococcum*, the down regulation of *Vrn-2* by short day is not followed by the up-regulation of the meristem identity gene *Vrn-1* until plants are transferred to long days. However, plants carrying the *Vrn-1lf* allele described above, or the *Vrn-1g* allele (34

bp deletion affecting the CARg box and no mutations in intron 1) show high levels of *Vrn-1* transcripts under short days (Fig. 2).

We interpret these results as evidence for the existence of a second *Vrn-1* repressor that is effective only under short days. In addition we hypothesize that the CARg box is the binding site of this short day repressor. Up-regulation of *Vrn-1* in accessions carrying mutations in the CARg-box resulted in an earlier initiation of spike development under short days. However, even in these genotypes, long days were required for a normal and timely heading time, suggesting the existence of additional regulatory control points downstream of the *Vrn-1* induction.



**Fig.2.** Transcript levels of *Vrn-1* under short days in F<sub>2</sub> plants segregating for the *Vrn-1f* and *Vrn-1g* alleles.

**The CARg box is the binding site of *TaVRT-2*.** It has been suggested before that *TaVRT-2* (*SVP-like* MADS-box protein) could be a vernalization repressor of *Vrn-1* through its interactions with the *Vrn-1* promoter<sup>10, 11</sup>. We confirmed that *TaVRT-2* can interact with the CARg box in the *Vrn-1* promoter by EMSA and identified (C/G)<sub>2</sub>(A/T)<sub>6</sub>(G/C)<sub>2</sub> as the optimal binding site using Random Binding Site Selection.

However, we failed to reproduce the results from Kane et al. (2005), which showed a down-regulation of *TaVRT-2* transcript levels in hexaploid wheat after long exposures to cold temperatures. Instead, we observed a significant up-regulation of *TaVRT-2* transcripts during vernalization under both short and long day conditions and both in spring and winter *T. monococcum* lines. These results are in agreement with a recent report in barley showing induction of *HvVRT-2* by vernalization<sup>12</sup>. These results, together with the previous demonstration that the CARg box is not required for the vernalization regulated repression of *Vrn-1*, suggest that *TaVRT-2* is unlikely to play an important role as a vernalization regulated repressor of *Vrn-1*.

It is possible that the observed interaction between *TaVRT-2* and the CARg box in the *Vrn-1* promoter plays a role later in the regulation of flower development. In Arabidopsis, *API* (homologue of *Vrn-1*) interacts with other MADS box proteins to confer sepal and petal identity after its initial role in shoot meristem identity<sup>13</sup>. In agreement with this hypothesis, ectopic expression of two *SVP-like* genes *BMI* and *BMI0* related to *HvVRT-2*

caused floral reversion phenotypes rather than changes in flowering time in barley<sup>12</sup>. These results suggest that *SVP-like* genes may be involved in the regulation of flower meristem identity rather than in the transition of the apex from vegetative to reproductive phases<sup>12</sup>. However, transgenic or mutant plants for *TaVRT-2* will be required to confirm or negate these hypotheses.

**Role of the *Vrn-1* first intron on epistatic interactions between *Vrn-1* and *Vrn-2*.** Strong epistatic interactions have been observed between *Vrn-1* and *Vrn-2* in both wheat and barley. However, EMSA and Chromatin Immuno-precipitation (ChIP) experiments failed to show a direct interaction between the VRN-2 protein and *Vrn-1* regulatory regions in the promoter or first intron suggesting an indirect interaction between these two genes.

Deletions or insertions in the *Vrn-1* first intron seem to have a stronger effect on the epistatic interactions between *Vrn-1* and *Vrn-2* than some of the mutations in the *Vrn-1* promoter. In the PI355515 x PI266844 *T. monococcum* population described above, no significant differences in flowering time were detected between *Vrn-2* alleles among the lines carrying the dominant *Vrn-1f* allele (intron insertion). The same result was observed in epistatic studies between these two genes in photoperiod sensitive barley lines, involving a *Vrn-H1* allele with a large deletion in the first intron<sup>7,14</sup>.

A different result was observed in a study involving a different *Vrn-1* allele with a 20-bp deletion upstream of the CArG box in the promoter region (*T. monococcum* accession G2528). In the population from the cross between G2528 and DV92 segregating for both *Vrn-1* and *Vrn-2*, the presence of the dominant *Vrn-1* allele with the 20-bp deletion reduced but did not eliminate the effect of *Vrn-2* allelic differences on flowering time<sup>15</sup>. Since the G2528 *Vrn-1* allele has no large insertions or deletions in the first intron, we hypothesize that the allelic variation at the *Vrn-1* first intron is more effective in suppressing the effect of *Vrn-2* on flowering time than the allelic variation in the region upstream of the CArG box in the *Vrn-1* promoter (Fig. 4).

In the epistatic interaction studies described above, the presence of recessive *vrn-2* alleles eliminated the effect of *Vrn-1* allelic variation on flowering time, independently of which dominant *Vrn-1* allele was present<sup>14,15</sup>. However, recent epistatic studies in barley<sup>7</sup> indicate that the presence of the photoperiod insensitive allele (*ppd-H1*) can modify these interactions (see Discussion).

**Natural variation in *Vrn-2* in tetraploid wheat.** In addition to the natural variation in *Vrn-1* regulatory regions described above, differences in the *Vrn-2* gene are also frequently associated with natural variation in vernalization requirement. Homozygous deletions or non-functional mutations of the *Vrn-2* gene result in a

spring growth habit in both diploid *T. monococcum* and barley<sup>8</sup>. Surprisingly, no allelic variation for this gene has been described so far in polyploid wheat. This is likely related to the fact that in a recessive *vrn-1* genetic background, simultaneous mutations at all the *vrn-2* homoeoalleles would be required to confer a spring growth habit. To facilitate the study of *Vrn-2* natural allelic variation in tetraploid wheat we developed a genetic stock carrying the recessive *vrn-A<sup>m</sup>2* allele from *T. monococcum* in the A genome and a deletion of the *Vrn-B2* locus (found in *T. dicoccon* accession PI 470739) in the B genome. We then generated plants heterozygous for both *Vrn-A2* and *Vrn-B2* in a genetic background carrying recessive *vrn-A1* and *vrn-B1* genes (from winter durum variety Durelle).

The F<sub>2</sub> population generated from this line showed segregation for a single vernalization gene (3:1 ratio of winter to spring lines). All lines carrying the dominant *Vrn-B2* allele from the durum varieties used in the crosses (Durelle and Langdon) had a winter growth habit whereas those homozygous for the *vrn-B2* deletion had a spring growth habit, regardless of the *Vrn-A2* allele. Lines homozygous for the *Vrn-B2* allele were significantly later than heterozygous *vrn-B2* suggesting partial dominance. Within the *vrn-B2* lines, lines carrying the *Vrn-A2* allele from *T. dicoccon* were as early as those homozygous for the non functional *vrn-A<sup>m</sup>2* allele. This result indicates that *T. dicoccon* carries a non-functional *vrn-A2* allele. The sequence of this allele revealed an R to C mutation in a conserved amino acid within the CCT domain (Fig. 3). We hypothesize that this mutation might be responsible for the lack of function of this allele. This R to C mutation was polymorphic in *T. dicoccoides* and *T. dicoccon* but was fixed in the durum accessions we tested.

Seq.#	Species	Function
1	<i>T. urartu</i> (A genome)	Winter
2	<i>Ae. tauschii</i> (D genome)	Winter
3	<i>T. monococcum</i> G1116	Winter
4	<i>T. monococcum</i> DV92	Spring
5	<i>T. turgidum</i> A genome	Spring

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1 EKRKRRRYDKQIRYESRKAYAELRPVNGRFVKV
2 EKRKRRRYDKQIRYESRKAYAELRPVNGRFVKV
3 EKRKRRRYDKQIRYESRKAYAELRPVNGRFVKV
4 EKRKRRRYDKQIRYESRKAYALRPVNGRFVKV
5 EKRKRRRYDKQIRYESRKAYAELRPVNGCFVKV

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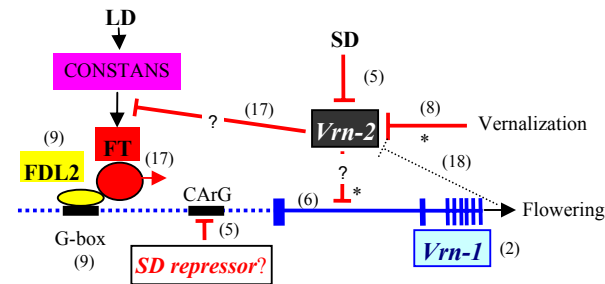
**Fig. 3.** Sequence alignment of the *Vrn-2* CCT domain from different Triticeae species. The R to W and R to C mutations are associated with recessive *vrn-2* alleles (loss of function).

These results confirmed that *Vrn-2* plays a significant role in establishing the vernalization requirement in polyploid winter wheat, and that natural allelic variation exists for both *Vrn-A2* and *Vrn-B2* in tetraploid wheat.

## DISCUSSION

A model is presented below to summarize our current understanding of the different interactions between flowering genes involved in the regulation of flowering time in wheat.

According to this model, *Vrn-2* is a repressor of flowering down-regulated by vernalization and short days<sup>5, 8, 16</sup>, which negatively regulates *Vrn-3* and *Vrn-1* (probably indirectly). *Vrn-3* is up-regulated by long days and promotes flowering by positively regulating *Vrn-1*<sup>17</sup> through its interactions with *TaFDL2*<sup>9</sup>. *TaFDL2* has the ability to bind *in vitro* with the *Vrn-1* promoter. The initiation of *Vrn-1* transcription is followed by the down-regulation of *Vrn-2*, as part of a feedback regulatory loop<sup>18</sup> (dotted line Fig. 4).



**Fig. 4.** Model for flowering interactions in wheat. Numbers in parenthesis indicate references for specific interactions. The dotted blue line indicates the *Vrn-1* promoter. G-box and CARG are regulatory sites. *Vrn-1* exons are represented by short vertical blue bars.  $\perp$  indicates repression and arrows indicate promotion. SD= Short day and LD= Long day. ?= putative intermediary genes. \*= differences with Trevaskis *et al.*<sup>19</sup> alternative model.

Unvernalized winter wheat plants grown under long days exhibit high levels of *Vrn-2* transcripts and low levels of *Vrn-1* and *Vrn-3*. Vernalization under long days results in the down-regulation of *Vrn-2*, and the up-regulation of *Vrn-3* and *Vrn-1*. Under short days all three genes show low transcript levels, but *Vrn-1* and *Vrn-3* are rapidly up-regulated after transfer to long days<sup>5</sup>. Mutations in the CARG box are associated with increased transcript levels of *Vrn-1* under short days, suggesting that this site might be recognized by a yet unidentified short day repressor

An alternative model proposed by Trevaskis *et al.*<sup>19</sup> differs from the one presented in Fig. 4 in the two interactions marked by \*. This alternative model proposes that the vernalization signal is perceived by *Vrn-1* rather than by *Vrn-2*, and that *Vrn-2* regulates *Vrn-1* only through its effect on FT. Critical experiments are still missing to validate one of these alternative models.

**Epistatic interactions:** The model presented in Fig. 4 provides a simple explanation for the epistatic interactions observed among these genes. In wheat

plants homozygous for non-functional *vrn-2* alleles (no repressor) and grown under long days, allelic differences in *Vrn-1* regulatory regions have no effect on flowering time<sup>14, 15</sup>. In addition, mutations in regulatory regions of *Vrn-1* and *Vrn-3* reduce or eliminate the effect of *Vrn-2* allelic differences on flowering time<sup>6, 17</sup>. We propose that these mutations preclude the recognition of *Vrn-1* regulatory regions by a *Vrn-2* mediated repressor, initiating the flowering regulatory cascade without vernalization.

The epistatic interactions described above can be affected by allelic differences at the *Ppd-1* locus, which is associated with major differences in response to photoperiod in both wheat and barley<sup>7</sup>. The barley *Ppd-H1* gene has been recently cloned and it was shown that the photoperiod insensitive *ppd-H1* allele alters the circadian expression of the photoperiod gene *CONSTANS* and reduces expression of its downstream target *FT*, delaying flowering under long days<sup>20</sup>. In barley lines carrying the recessive *vrn-H1* allele, the presence of *ppd-H1* insensitive alleles results in late flowering even when the *Vrn-H2* gene is deleted<sup>7</sup>. This confirms that part of the *Vrn-H2* effect on *Vrn-H1* is an indirect result of the *Vrn-H2* regulation of *FT* (Fig. 4). In the presence of the photoperiod insensitive *ppd-H1* allele lines with the *vrn-H1/vrn-H2* alleles flower late, but those with the *vrn-H1/Vrn-H2* alleles flower even later or fail to flower (B. Trevaskis personal communication). We interpret this as indirect evidence for a residual *Vrn-H2* repression of *Vrn-H1* independent of *FT* (Fig. 4). The *Ppd-1* epistatic interactions observed in barley cannot be easily translated to wheat, because of the different type of mutation present in the *Ppd-1* gene in these two species. In barley, an amino acid mutation in the conserved CCT domain likely results in a gene that cannot promote flowering efficiently under long days<sup>20</sup>. In wheat, the photoperiod insensitive *Ppd-D1a* allele has a 2 kb deletion in the promoter that is associated with misexpression of the *Ppd-D1* and induction of *FT* irrespective of daylength<sup>21</sup>. This results in dominant photoperiod insensitivity.

This model shows that multiple interconnections exist between photoperiod and vernalization in the regulation of flowering time in temperate cereals. In unvernallized plants grown under long day conditions there seems to be a continuous competition between the photoperiod pathway trying to activate *FT* and *Vrn-2* trying to repress it. Allelic variation at each of these loci may affect the final balance between these forces and have an effect on flowering time. We also showed here that natural allelic variation for *Vrn-2* also exists in polyploid wheat and that variation in the number of functional *Vrn-2* copies may have a significant impact on the final balance between flowering repression and promotion forces.

## ACKNOWLEDGEMENTS

This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant numbers 2007-35301-17737 and 2007-35301-18188 and by grant BID 1728/OC AR PICT 13442 from Argentina. A. Distelfeld was supported by a Vaadia-BARD Postdoctoral Fellowship Award No. FI-386-06.

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