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 AP1) 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 Immuno-precipitation (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 AP1, but is also affected by the paralogous MADS-box genes FUL and CAL. 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 5,4,3 and its first intron 5,7. In diploid Triticeae species such as T. monococcum and barley, natural allelic variation in vernalization requirement is also associated with the Vrn-2 locus 8. In these two diploid species Vrn-2 deletions or mutations in its coding region are associated with a recessive spring growth habit 8. 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 9. 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. In Arabidopsis, AP1 is expressed at high levels in the leaves only when FD is ectopically expressed in transgenic 35S::FD plants.

Regarding the CArG box regulatory site in the Vrn-1 promoter, it is not necessary for vernalization requirement. Based on natural allelic variation in the Vrn-A1 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. 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-A1 promoter, which includes the CArG box, and a recessive vrn-2 allele, which is known to confer a spring growth habit.

In the F2 population from the cross PI355515 with winter line G3116 (vrn-1 Vrn-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-A1f allele (spring), which has a 1bp deletion in the CArG box and a repetitive element inserted in the first intron. Lines homozygous for the recessive vrn-2 allele or carrying at least one copy of Vrn-A1f showed a strong winter 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-1f 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.

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, AP1 (homologue of Vrn-1) interacts with other MADS box proteins to confer sepal and petal identity after its initial role in shoot meristem identity. In agreement with this hypothesis, ectopic expression of two SVP-like genes BM1 and BM10 related to HvVRT-2
caused floral reversion phenotypes rather than changes in flowering time in barley. 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. 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, EMISA 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.

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. 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.

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. However, recent epistatic studies in barley indicate that the presence of the photoperiod insensitive allele (ppd-H1) can modify these interactions.

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. 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 homeoeoalleles 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-A1* 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 F2 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-A1* 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.

<table>
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<tr>
<th>Seq.#</th>
<th>Species</th>
<th>Function</th>
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<tbody>
<tr>
<td>1</td>
<td>T. urartu (A genome)</td>
<td>Winter</td>
</tr>
<tr>
<td>2</td>
<td>Ae. tauschii (D genome)</td>
<td>Winter</td>
</tr>
<tr>
<td>3</td>
<td>T. monococcum G1116</td>
<td>Winter</td>
</tr>
<tr>
<td>4</td>
<td>T. monococcum DV92</td>
<td>Spring</td>
</tr>
<tr>
<td>5</td>
<td>T. turgidum A genome</td>
<td>Spring</td>
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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 \(^5, 8, 16\), 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 \(^17\) through its interactions with TaFDL2 \(^9\). 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 \(^18\) (dotted line Fig. 4).

\[ \text{LD} \quad \text{CONSTANS} \quad \text{SD} \]

\[ \text{Vrn-1} \quad \text{FT} \quad \text{Vrn-2} \quad \text{Vrn-3} \]

\[ \text{G-box} \quad \text{CARG} \]

\[ \text{Flowering} \]

**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. ↓ indicates repression and arrows indicate promotion. SD= Short day and LD= Long day. *= putative intermediary genes. *= differences with Trevaskis et al. \(^19\) 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 \(^5\). 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. \(^19\) differs from the one presented in Fig. 4 in the two interactions marked by *\(^\ast\). 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 \(^10, 15\). 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 \(^6, 17\). 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 \(^7\). 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 \(^20\). 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 \(^7\). 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 allele ever 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 barely, an amino acid mutation in the conserved CCT domain likely results in a gene that cannot promote flowering efficiently under long days \(^20\). 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 \(^21\). 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 unvernalized 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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