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REGULATION OF FLOWERING TIME IN WHEAT

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Summary

Vernalization is the requirement of a long exposure to cold temperatures to induce flowering. In wheat and barley, the *VRN-1* and *VRN-2* genes are mainly responsible for this requirement. The alleles for winter growth habit are the ancestral forms of these genes in the Triticeae, and independent mutations in both genes have resulted in the recurrent generation of spring forms in the temperate cereals. The *VRN-1* gene is a meristem identity gene (≅ *APETALA1*) and is dominant for spring growth habit. Reduction of *VRN-1* transcript levels by RNA interference (RNAi) result in a delay in flowering of 2-3 weeks, suggesting that the transcript level of *VRN-1* is critical for the determination of flowering time in wheat.

In the wheat and barley lines characterized so far deletions in the promoter or the first intron of *VRN-1* result in a dominant spring growth habit, suggesting that these are important regulatory regions. Mutations in a single *VRN-1* copy in polyploid wheat are sufficient to determine a spring growth habit, but different combinations of dominant *Vrn-1* alleles result in different effects on flowering time. We propose that these mutations interfere with the recognition of a repressor coded or regulated by *VRN-2*.

The *VRN-2* gene is a zinc-finger CCT transcription factor and is dominant for the winter growth habit. This gene is down-regulated by vernalization, releasing the transcription of the *VRN-1* genes and promoting flowering. Mutations in the CCT domain or deletions of the *VRN-2* gene are associated with a spring growth habit in both barley and diploid wheat. Reduction of *VRN-2* transcript levels by RNAi in winter wheat variety Jagger resulted in the increase of *VRN-1* transcript levels and in a significant acceleration of flowering time.

In plants showing different combinations of dominant and recessive alleles we observed that the dominant *Vrn-1* alleles are expressed first and that the recessive *vrn-1* alleles are transcribed later. We have recently proposed the existence a feedback regulatory loop between *VRN-1* and *VRN-2* to explain these results. This coordinated regulation of dominant and recessive *VRN-1* alleles influences the timing of meristem differentiation and final flowering time.

We found here that the induction of the recessive alleles is mediated by the down-regulation of *VRN-2* after the initiation of transcription of the dominant *Vrn-1* allele. Once *VRN-2* is repressed, the recessive *vrn-1* alleles can be transcribed. Three experiments supporting this hypothesis are discussed.

Introduction

One critical trait in the adaptation of temperate grasses to cold winters is the requirement of long exposures to low temperatures (vernalization) to accelerate flowering. Vernalization requirement in temperate grasses is mainly controlled by allelic variation at the vernalization genes *VRN-1* and *VRN*-2 (Jensen et al. 2005;

vonZitzewitz et al. 2005; Yan et al. 2004b; Yan et al. 2003), which are different from the Arabidopsis genes with the same name (Gendall et al. 2001; Levy et al. 2002). The central repressors in the vernalization pathways of Arabidopsis (*FLC*, Michaels and Amasino 1999) and the temperate cereals (*VRN-2,* Yan et al. 2004b) belong to different gene classes and have no clear homologues in the other species suggesting an independent evolution of their vernalization pathways (Yan et al. 2004b).

The wheat *VRN-2* gene is a Zinc finger - CCT domain transcription factor (*ZCCT-1*) down-regulated by vernalization (Yan et al. 2004b). Non-functional mutations (*vrn-2a* allele) or complete deletions of this gene (*vrn-2b* allele) are associated with a recessive spring growth habit in diploid wheat (*Triticum monococcum* L.) and barley (*Hordeum vulgare* L.) (Dubcovsky et al. 2005; Yan et al. 2004b). Down-regulation of *VRN-2* by vernalization is followed by the up-regulation of the meristem identity gene *VRN-1* (similar to Arabidopsis *AP1,* Yan et al. 2003) in winter varieties from both species. These two genes show strong epistatic interactions suggesting that they are part of the same regulatory pathway (Dubcovsky et al. 2005; Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000). In spring lines homozygous for the non-functional *vrn-2* alleles, allelic differences in *VRN-1* have no effect on flowering time. Conversely, mutations in the promoter (Yan et al. 2004a; Yan et al. 2003) or the first intron (Fu et al. 2005) of the *VRN1* gene eliminate or reduce the effect of *VRN-2* and eliminate the vernalization requirement (Dubcovsky et al. 2005; Tranquilli and Dubcovsky 2000). The vernalization requirement is also eliminated, or greatly reduced, in transgenic winter plants of hexaploid wheat 'Jagger' (*T. aestivum* L.) when *VRN-2* transcript levels are reduced by RNA interference (RNAi::*VRN-2*) (Yan et al. 2004b).

Based on these results Yan et al. (2003, 2004) proposed a model in which *VRN-2* represses directly or indirectly the transcription of *VRN-1*. Down-regulation of *VRN-2* by vernalization releases *VRN-1* from this repression resulting in the induction of flowering. This model predicts that only the dominant *Vrn-1* allele will be transcribed in lines carrying simultaneously recessive and dominant alleles. We have recently confirmed this prediction in young isogenic lines of hexaploid wheat variety 'Triple Dirk' carrying different combinations of dominant and recessive *VRN-A1*, *VRN-B1*, and *VRN-D1* alleles, and also in heterozygous *VRN-1* diploid wheat plants (Loukoianov et al. 2005). Using the Triple Dirk near-Isogenic Lines (NIL) (Pugsley, 1971; Pugsley, 1972) we showed that in leaves of young plants only the dominant *Vrn-A1* alleles is transcribed in Triple Dirk D (TDD: *Vrn-A1 vrn-B1 vrn-D1*), only the *Vrn-B1*allele is transcribed in TDB (*vrn-A1 Vrn-B1 vrn-D1*), and only the *Vrn-D1* allele in TDE (*vrn-A1 vrn-B1 Vrn-D1*).

However, a few weeks later, transcripts from the recessive alleles were also detected in both the polyploid and heterozygous diploid spring plants suggesting the existence of a feedback regulatory loop that releases the recessive *vrn-1* alleles after the initiation of transcription of the dominant *Vrn-1* alleles (Loukoianov et al. 2005). In this paper we present three lines of evidence suggesting that this regulatory loop is mediated by the down-regulation of the *VRN-2* repressor after the initiation of the transcription of the dominant *VRN-1* alleles.

Materials and Methods

For the first experiment, RNA samples were collected from the leaves of diploid wheat *T. monococcum* winter accession G3116 (*vrn-A^m1*), spring accession PI 266844 (*Vrn-A^m1*, carrying a 1-bp deletion in the promoter *CArG* box), and F_2 heterozygous plants from the cross between these two accessions (Dubcovsky and Yan 2003; Yan et al. 2004b). These two accessions differ in an SNP in exon five that was used to develop a Cleavage Amplification Polymorphic Sequence (CAPS) marker to select heterozygous F_2 plants and to identify the parental source of the transcripts from the dominant *Vrn-A^m1* and recessive *vrn-A^m1* alleles in the heterozygous plants. The Quantitative PCR experiments were done using TaqMan systems developed before (Yan et al. 2004b; Yan et al. 2003).

The second experiment was performed using Near-Isogenic Lines (NIL) developed in hexaploid wheat variety 'Triple Dirk' (Pugsley 1971; Pugsley 1972). The winter isogenic line Triple Dirk C (TDC) has all three recessive *vrn-1* alleles, whereas the three spring Triple Dirk lines have one different dominant *Vrn-1* allele each (TDD: *Vrn-A1 vrn-B1 vrn-D1*; TDB: *vrn-A1 Vrn-B1 vrn-D1*, and TDE: *vrn-A1 vrn-B1 Vrn-D1*). In this experiment we used two SYBR GREEN® quantitative PCR systems to measure *VRN-1* and *VRN-2*. Quantification of the *VRN-1* transcripts was done by a combination of the conserved primers Ex4-5_F1 (TCAGATCCAGGAAGAACCAA) and Ex8_R1 (TTGATGTGGCT(A/C)ACCATC CA) which amplify all three *VRN1* copies. The quantification of the *VRN-A2* transcript levels was done with primers ZCCT-A1-F (GACCCATGGCTCACCTA GTG) and ZCCT-A1-R (TTGCTTCATTGCTAATAGTGTTGGT). We currently do not know the sequence of the *VRN-B2* and *VRN-D2* genes and, therefore, we are not sure if our *VRN-A2* primers amplify only the *VRN-A2* transcripts or a combination of the three different homoeoalleles present in hexaploid wheat.

In the third experiment we used transgenic Bobwhite plants (HWS common wheat) with reduced levels of endogenous *VRN1* caused by RNA interference (RNAi) (Loukoianov et al. 2005). Positive transgenic plants were confirmed by PCR of genomic DNA using primers Rs_S_F/R and Ri_AntiS_F/R designed from the vector sequence flanking the sense and antisense insertions (Yan et al. 2004b). We excluded the MADS-box and K-box domains from the cloned region to avoid interference with other MADS-box genes. Transcription levels of the endogenous *VRN-1* were investigated using a SYBR GREEN® quantitative PCR system using the conserved primers Ex4-5_F1 and Ex8_R1 AP1_Ex3 -F3 described above. The quantification of the *VRN-A2* transcript levels was done with primers ZCCT-A1-F and ZCCT-A1-R described above. Twelve transgenic plants and 12 controls at the third leaf stage were used in this experiment. Primers for the *ACTIN* endogenous controls were U211_SYBR_Actin_F: ACCTTCAGTTGCCCAGCAAT and U212_SYBR_Actin_R: CAGAGTCGAGCACAATACCAGTTG.

Quantitative PCR experiments were performed in an ABI7700 using *ACTIN* as endogenous controls (Yan et al. 2003). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate the *VRN-1* C_T values relative to the *ACTIN* endogenous controls. For the statistical analysis of the experiments comparing *VRN-1* and *VRN-2* transcript levels among different genotypes, we used a log transformation of the $2^{-\Delta\Delta C_T}$ values to correct the lack of homogeneity of variances in the untransformed data detected by Levene's test (SAS Institute Inc. 2003).

Results and Discussion

Diploid wheat experiment: For the diploid wheat experiment, we selected eight homozygous *vrn-A^{m}1*, seven homozygous *Vrn-A^{m}1*, and eleven heterozygous *vrn-A^{m}1 Vrn-A^{m}1* F_2 plants from the cross G3116 x PI 266844. First leaves from unvernalized plants carrying the dominant *Vrn-A^{m}1* alleles showed, as expected, significantly higher transcript levels of *VRN- A^{m}1* than the plants carrying the recessive *vrn-A^{m}1* alleles ($P < 0.0001$, Fig. 1A). The *VRN- A^{m}1* transcript levels in the heterozygous plants were closer to those in the homozygous *Vrn-A^{m}1* lines than to the ones observed in the homozygous *vrn-A^{m}1* lines (Fig. 1A).

The *VRN-2* transcript showed the opposite trend, with significantly lower levels of *VRN-2* transcripts in the homozygous *Vrn-A^{m}1* plants than in the homozygous *vrn-A^{m}1* plants ($P = 0.0001$). The heterozygous plants showed intermediate levels of *VRN-2* transcripts that were not significantly different from the average of the two homozygous classes (Fig. 1B).

The results from the *T. monococcum* F_2 plants segregating for the *VRN-A^{m}1* locus but homozygous for the dominant *Vrn-2* winter allele provide support for the hypothesis that the late induction of the recessive *vrn1* alleles is mediated by the down-regulation of *VRN-2*. A lower level of *VRN-2* transcripts was observed in the first leaves of the F_2 plants carrying one or two copies of the dominant *VRN-A^{m}1* allele relative to the plants homozygous for the recessive *vrn-A^{m}1* allele. All plants were grown in the same greenhouse under environmental conditions that generally do not result in down-regulation of *VRN-2* transcripts (no vernalization, 16 h light). The plants homozygous for the recessive *vrn-A^{m}1* allele served as controls for the normal transcript levels of *VRN-2* under our experimental conditions. We conclude that the initiation of the *Vrn-A^{m}1* transcription was likely the trigger leading to the observed decline in *VRN-A^{m}2* transcripts. The repression of the *VRN- A^{m}2* transcripts preceded the transcription of the *vrn-A^{m}1* alleles, providing a likely explanation to the later transcription of the recessive *vrn-A^{m}1* alleles.

Triple Dirk experiments: The isogenic Triple Dirk lines also showed opposite transcript levels of *VRN-1* and *VRN-2* in the first leaves of unvernalized plants. The overall *VRN-1* transcript level in TDD detected by the conserved primers (Ex4-5_F1 and Ex8_R1) was significantly higher ($P = 0.001$) than in TDB or TDE, which did not differ significantly between each other ($P = 0.40$, Fig. 1C). On the contrary, the *VRN-2* transcript levels in TDD were significantly lower ($P = 0.0009$) than in TDB or TDE, which did not differ significantly between each other ($P = 0.25$, Fig. 1D).

The first leaves of Triple Dirk isogenic line TDD which showed the highest levels of *VRN-1* transcripts, also showed the lowest levels of *VRN-2* transcripts. This suggests that transcription of the dominant *Vrn-A1* allele (directly or indirectly) represses *VRN-2*, and that the elimination of this flowering repressor then allows the initiation of the transcription of the recessive *vrn-B1* and *vrn-D1* alleles observed several days later.

RNAi transgenic Bobwhite experiments: The transgenic Bobwhite plants showed the expected reduction of the endogenous levels of *VRN-1* relative to the non-transgenic controls ($P=0.04$). The transcript levels of the endogenous *VRN-1* in the transgenic plants were less than half of the level observed in the non-transgenic controls (Fig. 1E).

The *VRN-2* transcript levels showed the opposite trend. A significant increase of the *VRN-2* transcript levels ($P<0.0001$) was observed in the transgenic plants relative to the non-transgenic controls. *VRN-2* transcript levels in the RNAi plants with reduced *VRN-1* levels were more than ten fold higher than in the control plants (Fig. 1F).

Conclusions: Taken together, these results provide a strong support to the hypothesis that the transcription of *VRN-2* is repressed after the initiation of the transcription of the dominant *VRN-1* alleles, resulting in the release of the recessive *vrn-1* from the *VRN-2* repression.

The proposed feedback regulatory loop starts with the *VRN-2* repression of the recessive *vrn-1* alleles. In the winter lines, vernalization represses *VRN-2* releasing all the *VRN-1* alleles, which then repress *VRN-2* transcription. In the spring wheat lines, the dominant *Vrn-1* alleles lack the correct recognition sites for the *VRN-2* repressor and are transcribed independently of the vernalization signal. After transcription of the *Vrn-1* alleles, *VRN-2* is repressed resulting in the later transcription of the recessive *vrn-1* alleles.

The induction of recessive *vrn-1* alleles after the transcription of the dominant *Vrn-1* alleles might contribute to the overall accumulation of VRN-1 products to levels that trigger the apex transition from the vegetative to the reproductive stage. One possible function of this feedback regulatory loop might be to coordinate the transcription of dominant and recessive alleles contributing to an earlier attainment of the inductive *VRN-1* transcript threshold, and triggering an irreversible flowering response.

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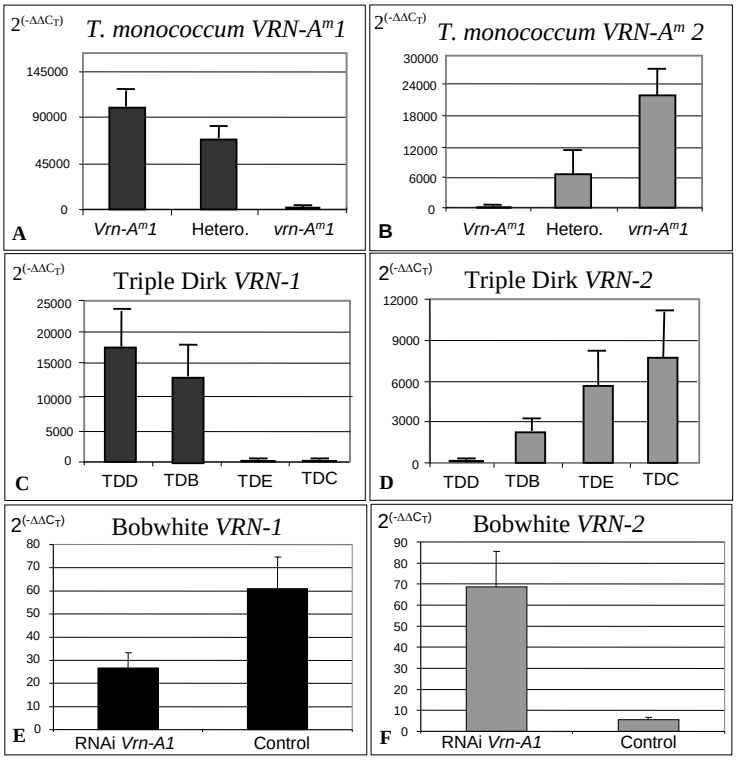

Figure 1. Comparison of *VRN-1* (A ,C, & E) and *VRN-2* (B, D, & F) transcript levels in the first leaves of unvernalized plants grown under 16 h light by Quantitative PCR. A & B) F_2 plants from the cross G3116 x PI 266844. Results are averages of 8 homozygous *Vrn-A^{m}1*, 11 heterozygous *vrn-A^{m}1 Vrn-A^{m}1*, and 7 homozygous *vrn-A^{m}1* plants. C & D) Isogenic Triple Dirk lines TDD (*Vrn-A1 vrn-B1 vrn-D1*), TDB (*vrn-A1 Vrn-B1 vrn-D1*), TDE (*vrn-A1 vrn-B1 Vrn-D1*) and TDC (*vrn-A1 vrn-B1 vrn-D1*). E&F) Transgenic RNAi Bobwhite plants with reduced levels of *VRN-1* transcripts compared to the non-transgenic control. Results are averages of 12 plants. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate the C_T values relative to the *ACTIN* endogenous control. Error bars represent one SE of the mean.