Regulation of VRN-1 Vernalization Genes in Normal and Transgenic Polyploid Wheat

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Vernalization, the requirement of a long exposure to low temperatures to accelerate flowering, is an essential adaptation of plants to cold winters. The vernalization gene VRN-1 plays an important role in this process in diploid (Triticum monococcum) and polyploid wheat (Triticum aestivum). We have recently shown that the diploid wheat VRN-A1 gene was similar to the Arabidopsis (Arabidopsis thaliana L. Heynh.) APETALA1 meristem identity gene. We also showed that dominant Vrn-A1 alleles were the result of loss-of-function mutations in regulatory regions recognized by a VRN-1 repressor, likely VRN-2. This model predicts that only the dominant Vrn-1 allele will be transcribed in lines carrying both recessive and dominant alleles. Here, we confirm this prediction in young isogenic lines of hexaploid wheat carrying different dominant Vrn-A1, Vrn-B1, and Vrn-D1 alleles, and also in heterozygous VRN-1 diploid wheat plants. However, a few weeks later, transcripts from the recessive alleles were also detected in both the polyploid and heterozygous diploid spring plants. Transcription of the recessive alleles was preceded by a reduction of the transcript levels of VRN-2. These results suggest that the dominant Vrn-1 allele or a gene regulated by VRN-1 down-regulates the VRN-2 repressor facilitating the transcription of the recessive alleles in unvernalized plants. We also show here that the level of VRN-1 transcripts in early developmental stages is critical for flowering initiation. A reduction of VRN-1 transcript levels by RNA interference delayed apex transition to the reproductive stage, increased the number of leaves, and delayed heading time by 2 to 3 weeks. We hypothesize that the coordinated transcription of dominant and recessive alleles may contribute to an earlier attainment of the VRN-1 transcript level threshold required to trigger flowering initiation in polyploid wheat.

Common wheat (Triticum aestivum) was one of the first crops domesticated by man and, since then, has traveled with human populations to a wide range of environments. Wheat adaptability to this wide range of environments is partially due to the exploitation of genetic variation in daylength sensitivity and vernalization requirement, which provide a flexible regulation of flowering time. Vernalization, the requirement of a prolonged exposure to low temperatures to accelerate flowering, is particularly important for fall-planted wheat varieties to prevent flower development during winter and protect the environmentally sensitive floral organs. Wheat varieties that require vernalization to flower are referred to as winter wheats, whereas those without a vernalization requirement are identified as spring wheats.

Allelic variation at the VRN-1 locus is one of the main sources of genetic differences in vernalization requirement in both diploid (Triticum monococcum) and polyploid wheats (for review, see McIntosh et al., 2003). Common wheat is a hexaploid species (2n = 42, genomes AABBDD) and carries three homoeologous copies of the VRN-1 gene, one in each of the three genomes, which are designated VRN-A1, VRN-B1, and VRN-D1 (McIntosh et al., 2003). A dominant (Vrn) allele at any one of these loci is sufficient to confer a spring growth habit (Stelmakh, 1987).

We have recently used diploid wheat (2n = 14, genome A′A′A′) to clone the VRN-A′A′1 vernalization gene using a positional cloning approach (Yan et al., 2003). We demonstrated that VRN-A′A′1 is the wheat ortholog of the Arabidopsis (Arabidopsis thaliana L. Heynh.) meristem identity gene APETALA1 (AP1) and that mutations in the VRN-1 promoter region (Dubcovsky and Yan, 2003; Yan et al., 2003, 2004a) or in the first intron (Fu et al., 2005) were completely linked to spring growth habit in diploid and polyploid wheat. Additional variation in vernalization requirement in diploid wheat is determined by VRN-2, a Zinc finger-CCT domain transcription factor (ZCCT1) down-regulated by vernalization (Yan et al., 2004b). Non-functional mutations or complete deletions of this gene are associated with a recessive spring growth habit in wheat and barley (Hordeum vulgare; Yan et al., 2004b). VRN-2 represses flowering likely by repressing VRN-1 (Yan et al., 2003, 2004b).

The Arabidopsis ortholog to the wheat VRN-1 gene (AP1) is not directly regulated by vernalization, and no natural or induced allelic variation at this locus has been found to be associated with differences in
vernalization requirement in this species (Gazzani et al., 2003). An additional difference between wheat and Arabidopsis AP1 genes is the tissue specificity of the AP1 transcripts. In Arabidopsis AP1 transcription is restricted to apices, whereas in diploid wheat it is transcribed in both leaves and apices (Yan et al., 2003). In both tissues, vernalization accelerates the initiation of VRN-1 transcription in winter wheat accessions (Yan et al., 2003).

A similar up-regulation of VRN-1 transcription by vernalization was reported in leaves of hexaploid wheat (Danyluk et al., 2003; Trevaskis et al., 2003). However, these studies did not differentiate among the multiple copies of the VRN-1 genes present in the hexaploid genome of bread wheat. A useful tool to study the effect of the different VRN-1 genes present in polyploid wheat without the confounding effect of other genes affecting flowering time is the set of near-isogenic lines developed in hexaploid wheat variety Triple Dirk (Pugsley, 1971, 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 (Triple Dirk D [TDD]: Vrn-A1 vrn-B1 vrn-D1; Triple Dirk B [TDB]: vrn-A1 Vrn-B1 vrn-D1, and Triple Dirk E [TDE]: vrn-A1 vrn-B1 Vrn-D1).

In the Triple Dirk spring isogenic lines and in most of the spring hexaploid varieties (Stelmakh, 1987; Fu et al., 2005), there is a mixture of dominant and recessive alleles within the same nucleus, and their relative transcription levels in different genetic backgrounds are unknown. Based on the diploid wheat results, we proposed a model for the regulation of VRN-A1, in which the dominant alleles were the result of loss-of-function mutations in regulatory regions recognized by a VRN-1 repressor (Yan et al., 2003). This model predicts that only the dominant Vrn-1 alleles will be transcribed in unvernalized lines carrying both recessive and dominant alleles.

In this study, we characterize the transcription levels of individual dominant and recessive VRN-A1, VRN-B1, and VRN-D1 genes in different Triple Dirk isogenic lines to test the previous prediction of the model. Utilizing RNA interference, we also test the hypothesis that reduction of VRN-1 transcript levels will delay flowering initiation in polyploid wheat.

RESULTS

**Genome-Specific VRN-1 Transcript Level Changes during Development**

We first confirmed that VRN-1 was transcribed in leaves of the unvernalized spring isogenic lines of Triple Dirk but not in the winter isogenic line using reverse transcription (RT)-PCR and conserved primers, which amplified transcripts from VRN-A1, VRN-B1, and VRN-D1. Digestion of the RT-PCR products from the spring line TDD with restriction enzyme HindIII demonstrated that only the dominant Vrn-A1 allele (283 + 30-bp fragments) was transcribed at the first-leaf stage (Fig. 1A). VRN-B1 and VRN-D1 transcripts have an additional HindIII site (173 + 110 + 30-bp) and were not detected in TDD at this developmental stage (Fig. 1A). At the second- and third-leaf stages, transcripts from the recessive vrn-B1 and vrn-D1 alleles were hardly detected in TDD, but their abundance continued to increase and, by the time the plants reached six leaves, were easily detected (Fig. 1B).

Similar results were observed for the dominant VRN-1 alleles in TDB and TDE, but transcripts were detected later than in TDD (Fig. 1A). In plants at the second- and third-leaf stages, we detected transcripts only from the dominant Vrn-B1 allele in TDB and from the Vrn-D1 allele in TDE (Fig. 1A). To differentiate the VRN-B1 and VRN-D1 transcripts, we digested the RT-PCR products from TDB and TDE at the third-leaf stage with restriction enzyme Als1 and separated the digestion products in a 6% polyacrylamide gel. The 47-bp band characteristic of the VRN-D1 gene was not detectable in the TDB transcripts, and the 156-bp band characteristic of the B genome was not detectable in the TDE transcripts (Fig. 1D). These results confirmed that at the third-leaf stage, transcripts from the dominant Vrn-D1 allele were predominant in TDE and transcripts from the dominant Vrn-B1 allele were predominant in TDB. However, transcripts from the recessive alleles were clearly detectable by the time the TDB and TDE plants had six leaves (Fig. 1B).

In the previous experiments, the controls for each of the dominant Vrn-1 alleles were the other two spring isogenic lines carrying the recessive vrn-1 allele corresponding to that locus. We also compared the spring lines with the unvernalized TDC winter plants to see if the early expression of the recessive alleles observed in the spring lines was also observed in the winter isogenic line. Unvernalized TDC plants showed no vrn-1 transcripts in leaves from plants at the first-leaf stage, the sixth-leaf stage (Fig. 1, A and B), or even 2 months later (data not shown). This indicates that the transcription of the dominant Vrn-1 alleles was a necessary step to accelerate the transcription of the recessive alleles in the unvernalized plants.

Under our greenhouse conditions, winter TDC plants flowered several months later than the spring lines. We collected RNA samples from flowering TDC flag leaves and compared them to samples collected previously from flag leaves of the three spring isogenic lines. The abundance of the VRN-A1 (not digested) transcripts relative to that of the VRN-B1 and VRN-D1 (HindIII-digested) transcripts within each sample was similar in the winter TDC and the three spring TDD, TDB, and TDE lines at flowering time (Fig. 1C).

**Quantitative Analyses of VRN-A1, VRN-B1, and VRN-D1 Transcript Levels**

To quantify the differential expression of the VRN-1 genes from the three genomes in the early
developmental stages of the different spring isogenic lines, we developed quantitative PCR systems that preferentially amplified VRN-1 genes from each genome. Amplification of DNA from bacterial artificial chromosomes (BACs) containing the VRN-A1, VRN-B1, and VRN-D1 genes with the three different sets of primers confirmed their genome specificity (Fig. 2, A–C).

The quantitative PCR experiment was done with TDD, TDB, and TDE isogenic lines at the fourth-leaf stage using ACTIN as an endogenous control (Yan et al., 2003). With the VRN-A1-specific primers, we detected a significantly higher proportion of transcripts from the dominant Vrn-A1 allele in TDD relative to the recessive vrn-A1 alleles from TDB and TDE (Fig. 2D). When we used the VRN-B1-specific primers, we detected a significantly higher proportion of transcripts from the dominant Vrn-B1 allele in TDB relative to the recessive vrn-B1 alleles from TDD and TDE (Fig. 2E). Finally, when we used the VRN-D1-specific primers, we found that TDE has higher levels of transcripts from the dominant Vrn-D1 allele than from the recessive vrn-D1 alleles from TDB and TDD (Fig. 2F). The quantitative PCR experiments confirmed the RT-PCR results, indicating that at the early developmental stages most of the RNA transcripts were originated from the dominant Vrn-1 alleles.

When comparing the transcription levels at the fourth-leaf stage in each isogenic line using the primers for its respective dominant allele (Fig. 2G), we found that the different VRN-1 genes had inherently different transcription levels in leaves. To test if these differences were also present after vernalization, we analyzed the transcript levels of each of the recessive vrn-1 alleles before and after vernalization in the winter TDC. This experiment, including five to six plants for each allele, showed a similar result to the one observed for the dominant alleles in the spring lines: The VRN-A1 transcript levels were significantly higher than those from VRN-B1 ($P = 0.05$), and the VRN-B1 transcript levels were significantly higher than the VRN-D1 transcript levels ($P = 0.03$) in the vernalized plants (Fig. 2H). No significant levels of vrn-1 transcripts were detected in the unvernalized winter plants.

**VRN-1 Transcription in Apical Meristems**

Analysis of pools of apices from TDD at the first-leaf stage revealed transcripts only for the dominant Vrn-A1 allele but not from the recessive vrn-B1 and vrn-D1 alleles (Fig. 1E). However, when apices were collected from TDD plants at the fourth-leaf stage, we detected VRN-1 transcripts from all three genomes (Fig. 1F). These results confirmed that the earlier
transcription of the dominant Vrn-1 alleles observed in the leaves also occurred in the apices.

**VRN-1 Transcription in Heterozygous Diploid Wheat**

To determine if the earlier transcription of dominant Vrn-1 alleles relative to the recessive vrn-1 alleles was limited to the polyploid wheat species, we characterized the VRN-1 transcription in heterozygous VrnAm1vrnAm1 diploid wheat plants at different developmental stages. Heterozygous plants were selected from F2 plants from the cross between winter accession G3116 (vrn-Am1) and spring accession PI 266844 (Vrn-Am1) using molecular markers (see “Materials and Methods”).

At the one-leaf and three-leaf stages, digestion of the 277-bp amplification products revealed that all transcripts had the three Hinfl sites characteristic of the dominant Vrn-Am1 allele (8 + 20 + 85 + 164 bp). However, at the sixth-leaf stage both dominant and recessive alleles were detected after Hinfl digestion of the VRN-1 amplification products (8 + 20 + 85 + 164 + 249 bp; Fig. 1G).

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**VRN-2 Transcript Levels in Genotypes with Different VRN-1 Alleles**

Our current model for the epistatic interactions between VRN-1 and VRN-2 genes suggest that VRN-2 is a repressor of VRN-1 (Tranquilli and Dubcovsky, 2000; Yan et al., 2003). Therefore, the simplest explanation for the transcription of the recessive vrn-1 allele was that VRN-2 was repressed after the initiation of the transcription of the dominant VRN-1 alleles. To test this hypothesis, we performed two additional experiments in diploid and polyploid wheat.

For the diploid wheat experiment, we selected eight homozygous vrn-A”1, seven homozygous Vrn-A”1, and 11 heterozygous vrn-A”1 Vrn-A”1 F2 plants from the cross G3116 × PI 266844. First leaves from vernalized plants carrying the dominant Vrn-A”1 alleles showed, as expected, significantly higher transcript levels than the plants carrying the recessive vrn-A”1 alleles (P < 0.0001; Fig. 3A). The VRN-1 transcript levels in the heterozygous plants were closer to those in the homozygous Vrn-A”1 lines than to the ones observed in the homozygous vrn-A”1 lines,
Figure 3. Comparison of VRN-1 (A and C) and VRN-2 (B and D) transcript levels in the first leaves of unvernalized plants grown under 16 h light. A and B, F, plants from the cross G3116 × PI 266844. Results are averages of eight homozygous Vrn-Am1, 11 heterozygous vrn-Am1 Vrn-A1, and seven homozygous vrn-A1 plants. VRN-A1 genotypes were determined using the Tm_Ex4_F/Tm_Ex7_R cleaved amplified polymorphic sequence marker. C and D, Iso- genic 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). Error bars represent one SE of the mean.

showing a significant departure from a linear response (quadratic contrast \( P = 0.003 \); Fig. 3A). The VRN-2 transcript showed the opposite trend, with significantly lower levels of VRN-2 transcripts in the homozygous Vrn-A1 plants than in the homozygous vrn-A1 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 (quadratic contrast \( P = 0.77 \); Fig. 3B).

The isogenic Triple Dirk lines also showed contrasting 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 from each other (\( P = 0.40 \); Fig. 3C). 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 from each other (\( P = 0.25 \); Fig. 3D).

Taken together, these results suggest that transcription of VRN-2 was 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.

RNA Interference of VRN-1 Delays Flowering Time

In order to investigate the correlation between VRN-1 transcript levels and flowering time, we transformed the hexaploid spring wheat variety Bobwhite (dominant Vrn-A1) with an RNAi construct containing the 5' end from the VRN-A1 gene. Since the region chosen for RNA interference is conserved among all the VRN-1 alleles sequenced so far, we expected all VRN-1 transcripts to be affected by the RNAi. We identified two independent transgenic plants by PCR of genomic DNA. One of them, J88-255b, showed the expected delay in heading time relative to the nontransgenic controls. The other one, J88-186, flowered at the same time as the control. The presence of the transgene was confirmed in both lines by Southern blots using a probe for the 35S promoter. Two positive restriction fragments were observed in J88-255b and four in J88-186, and these fragments cosegregated in the progeny of each transgenic plant (data not shown).

We self-pollinated one positive T1 plant from each transgenic line and determined the presence or absence of the transgene in 16 T2 plants from J88-255b (nine nontransgenic and seven transgenic) and 16 T2 plants from J88-186 (eight nontransgenic and eight transgenic). Quantitative PCR analysis of the unvernalized T2 plants showed a reduction in the endogenous levels of total VRN-1 transcripts in the transgenic plants relative to the nontransgenic siblings for J88-255b (\( P < 0.05 \); Fig. 4A) but not for J88-186 (\( P = 0.51 \)). These results suggest that the J88-186 positive plants contain nonfunctional transgene(s) and explain the absence of differences in heading time between the transgenic and nontransgenic J88-186 plants (\( P = 0.36 \)).

Among the progeny of the J88-255b transgenic plant, those carrying the transgene headed, on average, 14 d later than the nontransgenic plants (\( P < 0.001 \); Fig. 4B). An additional experiment using 84 T3 plants showed an average of 19 d delay in heading time in the transgenic plants relative to their nontransgenic sister lines (\( P = 2.7 \times 10^{-22} \)). In addition, the transgenic plants showed an average of 2.7 more total leaves than the nontransgenic controls (\( P = 3.2 \times 10^{-10} \); Fig. 4C). These results confirmed that a reduction in the VRN-1 transcript levels correlated with a delay in flowering initiation.

DISCUSSION

Validation of the Model for the Regulation of VRN-1 Transcription

The VRN-1 gene is dominant for spring growth habit, and its transcription is gradually up-regulated
in winter wheat during vernalization (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). On the contrary, the vernalization gene $VRN-2$ is dominant for winter growth habit, and its transcription is down-regulated during vernalization (Yan et al., 2004b). Significant epistatic interactions between these two genes in diploid wheat indicated that they were part of the same regulatory pathway. The alleles for spring growth habit ($Vrn-1$ and $vrn-2$) were epistatic to the alleles for winter growth habit ($vrn-1$ and $Vrn-2$), and, therefore, only the double homozygous $vrn-1/Vrn-2$ plants showed a winter growth habit (Tranquilli and Dubcovsky, 2000). In hexaploid wheat, most of the allelic variation in vernalization requirement occurs at the $VRN-1$ locus, and significant epistatic interactions occur among the $VRN-1$ copies located in the three genomes. One dominant $Vrn-1$ allele at any of the three homoeoloci is sufficient to determine a spring growth habit (Stelmakh, 1993).

To explain the observed dominance and epistatic interactions, we proposed a model in which $VRN-1$ is repressed directly or indirectly by $VRN-2$ (Yan et al., 2003). When $VRN-2$ is down-regulated during vernalization, $VRN-1$ is released from its repression and initiates the flowering regulatory cascade (Fig. 5). According to this model, a single $VRN-1$ allele not repressed by $VRN-2$ is sufficient to induce flowering, resulting in a dominant spring growth habit. The black rectangle on top of the dominant $Vrn-A1$ allele in Figure 5 represents the disruption of the recognition site at the $VRN-1$ locus. All the dominant $Vrn-1$ alleles sequenced so far differ from their respective recessive alleles by mutations in regulatory regions within the promoter (Dubcovsky and Yan, 2003; Yan et al., 2003, 2004a) or the first intron (Fu et al., 2005) rather than by mutations in the coding region. These results suggest that spring wheat varieties avoid suppression of $VRN-1$ because they lack a regulatory sequence that interacts with a vernalization-sensitive flowering repressor (likely $VRN-2$).

One prediction of this model is that only the transcripts from the dominant alleles would be observed in unvernalized plants of polyploid wheat carrying different combinations of dominant and recessive $VRN-1$ alleles. In this report, we confirm this prediction, but only for young plants. At the beginning of the $VRN-1$ transcription we only detected transcripts from the dominant $Vrn-1$ allele present in TDD, $Vrn-B1$ allele in TDB, and $Vrn-D1$ allele in TDE (Fig. 1A).

These results also confirmed the identity between $VRN-1$ and $AP1$ for each of the three $VRN-1$ homoeologous genes in hexaploid wheat, as previously observed in diploid wheat (Yan et al., 2003). There was a perfect correspondence between the dominant $Vrn-1$ allele present in TDD, TDB, and TDE and the $AP1$ gene that was transcribed earlier in each line.

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**Figure 4.** Characterization of transgenic Bobwhite wheat plants carrying a $VRN-1$ RNAi construct. A, $VRN-1$ transcript levels. B, Flowering time. C, Total number of leaves. Results are averages of seven to nine (A and B), or 37 to 39 plants (C). Error bars represent one SE of the mean.

**Figure 5.** Hypothetical model to explain the developmental progress in transcription initiation of dominant and recessive $VRN-1$ alleles in polyploid wheat TDD ($Vrn-A1 vrn-B1 vrn-D1$). Genes are in white boxes and proteins in gray boxes. Thin arrows indicate transcription/translation and “L” indicates repression. The black rectangle and dotted bar above the dominant $Vrn-A1$ allele indicate the lack of interaction with the $VRN-2$ repressor. According to this model, the initiation of transcription of the dominant $Vrn-1$ allele results in the direct or indirect down-regulation of $VRN-2$ transcription. As development progresses, the absence of the $VRN-2$ repressor allows transcription of the recessive $vrn-1$ alleles. Accumulation of the $VRN-1$ protein above a critical level triggers the transition to flowering.
Transcription of Dominant \textit{Vrn}-1 Alleles Accelerates Transcription Initiation of Recessive \textit{vrn}-1 Alleles in Unvernalized Plants

After the TDD, TDB, and TDE plants reached the third-leaf stage, transcripts from the recessive \textit{vrn}-1 homoeoalleles began to accumulate, although at lower levels than those from the dominant homoeoalleles (Fig. 1A). By the time the plants had six leaves, transcript levels from both the dominant and recessive alleles were clearly detected. Nonvernalized winter plants (TDC) showed no detectable transcripts for any of the \textit{VRN}-1 genes for several months. This result indicates that the transcription of the dominant allele was a necessary step to accelerate the transcription of the recessive \textit{vrn}-1 alleles in unvernalized plants.

We interpret this result as evidence of the existence of an interaction between \textit{VRN}-1 or an intermediate factor regulated by \textit{VRN}-1, with one or more repres- sors of the recessive \textit{vrn}-1 alleles (e.g. \textit{VRN}-2).

The acceleration of the transcription initiation of the recessive alleles after the transcription of the dominant \textit{Vrn}-1 alleles was observed both in leaves and apices, indicating that the gene(s) implicated in this process should be expressed in both tissues. Therefore, it is unlikely that this will be a gene that acts late in the development of the spike and flowers, since most such genes are not expressed in the leaves (e.g. \textit{AGLG1}; Yan et al., 2003). It is interesting to point out that in both Arabidopsis and rice (\textit{Oryza sativa}) the \textit{VRN}-1 orthologous \textit{API} genes are transcribed in the apices but not in the leaves (Mandel et al., 1992; Lim et al., 2000).

TDC and several other winter wheats do not have an absolute requirement for vernalization and eventually flower even in the absence of vernalization. We showed here that when the winter TDC plants flowered in the absence of vernalization, transcripts from all three recessive \textit{vrn}-1 alleles were present in the flag leaves (Fig. 1C).

Acceleration of Recessive \textit{vrn}-1 Alleles Transcription in Heterozygous Diploid Wheat

To determine if the acceleration of the transcription initiation of the recessive alleles by the transcription of the dominant alleles was a particular mechanism that evolved in polyploid wheat species to coordinate the transcription of the different \textit{VRN}-1 copies present in their multiple genomes, we explored the transcription profiles of dominant and recessive \textit{VRN}-1 alleles in diploid wheat. Heterozygous \textit{vrn}-A\textsuperscript{1}Vrn-A\textsuperscript{1} plants are equivalent to polyploid wheat plants with recessive and dominant alleles in the different genomes. In both cases, dominant and recessive alleles are present simultaneously within the same nucleus.

In the young heterozygous diploid wheat plants (first- to third-leaf stage), we observed transcripts only from the dominant \textit{Vrn}-A\textsuperscript{1} allele, but several days later the recessive \textit{vrn}-A\textsuperscript{1} allele transcripts started to appear, and at the sixth-leaf stage both types of transcripts were detected (Fig. 1G). Winter diploid wheat plants homozygous for the recessive \textit{vrn}-A\textsuperscript{1} alleles showed no \textit{VRN}-A\textsuperscript{1} transcripts even 4 months later.

The presence of similar transcription patterns in diploid and polyploid wheat indicates that the acceleration of the transcription initiation of the recessive alleles after the dominant alleles is not a mechanism that evolved in polyploid wheat. Based on these results, it seems more likely that the polyploid species used a mechanism that was already present in their diploid ancestors to coordinate the transcription of the multiple \textit{VRN}-1 copies present in their genomes.

The Acceleration of the Transcription of the Recessive \textit{vrn}-1 Alleles Is Likely Mediated by the Down-Regulation of \textit{VRN}-2 Transcription

The observed acceleration of the accumulation of transcripts from the recessive \textit{vrn}-1 alleles after the initiation of transcription of the dominant \textit{Vrn}-1 alleles can be explained by two mutually exclusive hypotheses. The simplest one is that the transcription of a dominant \textit{Vrn}-1 allele results in the direct or indirect down-regulation of \textit{VRN}-2, releasing the recessive \textit{vrn}-1 alleles from their repressor (Fig. 5). The alternative hypothesis is that the transcription of the recessive \textit{vrn}-1 alleles is not mediated by the repression of \textit{VRN}-2.

The results from the diploid wheat \textit{F\textsubscript{2}} plants segregating for the \textit{VRN}-A\textsuperscript{1} locus but homozygous for the dominant \textit{Vrn}-2 winter allele provide support for the first hypothesis. A lower level of \textit{VRN}-2 transcripts was observed in the first leaves of the \textit{F\textsubscript{2}} plants carrying one or two copies of the dominant \textit{VRN}-A\textsuperscript{1} allele relative to the plants homozygous for the recessive \textit{vrn}-A\textsuperscript{1} allele. All plants were grown in the same greenhouse under environmental conditions that generally do not result in reduction of \textit{VRN}-2 transcripts (no vernalization, 16 h light). The plants homozygous for the recessive \textit{vrn}-A\textsuperscript{1} allele served as controls for the normal transcript levels of \textit{VRN}-2 under our experimental conditions. We conclude that the initiation of the \textit{Vrn}-A\textsuperscript{1} transcription was likely the trigger leading to the observed decline in \textit{VRN}-A\textsuperscript{2} transcripts. The repression of the \textit{VRN}-A\textsuperscript{2} transcripts preceded in time the transcription of the \textit{vrn}-A\textsuperscript{1} alleles, providing a likely explanation to the later transcription of the recessive \textit{vrn}-A\textsuperscript{1} alleles.

A similar trend was observed in the first leaves of Triple Dirk isogenic lines, where TDD showed the highest levels of \textit{VRN}-1 transcripts and the lowest levels of \textit{VRN}-2 transcripts. The model presented in Figure 5 for TDD proposes that transcription of the dominant \textit{Vrn}-A\textsuperscript{1} allele (directly or indirectly) represses \textit{VRN}-2 and that the elimination of this flowering repressor then allows the initiation of the transcription of the recessive \textit{vrn}-B\textsuperscript{1} and \textit{vrn}-D\textsuperscript{1} alleles.

One point that still requires additional research is the observed delay between the reduction of the \textit{VRN}-2 transcript levels and the induction of the recessive \textit{vrn}-1 alleles. One possible explanation for this
time lag could be the persistence of the VRN-2 protein after the repression of the VRN-2 transcription. An alternative explanation could be that at the start of the plant life cycle, certain VRN-1 alleles, under the influence of VRN-2, are silenced by chromatin modifications that are possibly similar to those reported for the silencing of FLC after vernalization (Gendall et al., 2001; Levy et al., 2002; He et al., 2003; Bastow et al., 2004; Sung and Amasino, 2004). The lag may be due to a requirement for several mitotic cycles to erase these repressive modifications and substitute active modifications in the VRN1 chromatin.

Effect of VRN-1 Transcript Levels on Flowering Initiation

Our results indicate that the level of VRN-1 transcripts plays a critical role in the induction of flowering and that this could explain several observations of variation in flowering time among different genetic stocks. For example, the higher transcript levels of VRN-A1 relative to VRN-B1 and VRN-D1 offers a simple explanation for the stronger effect of the dominant Vrn-A1 allele in reducing its vernalization requirement relative to the other two VRN-1 alleles (Halloran, 1967; Kato and Yamagata, 1988; Trevaskis et al., 2003). Additional support for the importance of VRN-1 transcript levels in the initiation of flowering comes from early studies using Chinese Spring cytogenetic stocks. Plants of Chinese Spring tetrasomic 5D lines with four copies of the dominant Vrn-D1 allele flower earlier and have a reduced number of leaves compared to the disomic plant with two Vrn-D1 copies (Halloran, 1967). Unvernalized plants of Chinese Spring tetrasomic 5D line have the same number of leaves at heading as the vernalized Chinese Spring, indicating no requirement for several mitotic cycles to erase these repressive modifications and substitute active modifications that are possibly similar to those reported for the silencing of FLC after vernalization (Gendall et al., 2001; Levy et al., 2002; Bastow et al., 2004; Sung and Amasino, 2004). The lag may be due to a requirement for several mitotic cycles to erase these repressive modifications and substitute active modifications in the VRN1 chromatin.

Regulation of VRN-1 Vernalization Genes in Wheat

MATERIALS AND METHODS

Plant Materials

Dr. Kim Kudwell (Washington State University, Pullman, WA) provided seeds of the isogenic lines of Triple Dirk originally developed by Pugsley (1971, 1972). TDC has a winter growth habit and recessive alleles at the three VRN-1 genes (vra1vrb1vrd1). The other three isogenic lines have a spring growth habit determined by single VRN-1 alleles. TDD is dominant for the Vrn-A1 allele (vra1vrb1vrd1), TDB is dominant for the Vrn-B1 allele (vra1vrb1vrd1), and TDE is dominant for the Vrn-D1 allele (vra1vrb1vrd1).

Sequences

The complete sequences of the coding regions of the VRN-A1 genes were obtained from BACs 1256C17 (Triticum turgidum, AY747598) and 231A16 (Triticum monococcum, AY188331) and from genomic DNA of TDC (vra1vrb1vrd1, AY747600; Fu et al., 2005). Sequences for the VRN-B1 gene were obtained from BAC 1225D16 (T. turgidum, AY747602) and from genomic DNA of TDC (vra1vrb1vrd1, AY747604) and TDB (vra1vrb1vrd1, AY747603; Fu et al., 2005). Sequences for the VRN-D1 gene were obtained from TDC (vra1vrb1vrd1, AY747606) and TDE (vra1vrb1vrd1, AY747597; Yan et al., 2003; Sherman et al., 2004; Fu et al., 2005). VRN1 sequences from cDNAs AY280870 (Triticum aestivum, VRN-B1) and AB007504 (T. aestivum, VRN-D1) were identical to the respective regions in the B and D genomic DNAs. The complete cDNA sequence for VRN-A1 was obtained from the combination of expressed sequence tags CD936812 and B531225 (T. aestivum, VRN-A1).

RT-PCR Experiments

RNA samples were extracted using the TRIZOL method (Invitrogen, Carlsbad, CA; Yan et al., 2003) from leaves of TDD, TDB, TDE, and TDC unvernalized plants at different developmental stages, defined by the number of completely emerged leaves. Samples were also collected from TDD apices at the first- and fourth-leaf stages. Approximately 50 to 100 apices were pooled per RNA extraction. Plants were grown in the greenhouse (20°C–27°C) under long-day conditions (16 h light). The vernalization of the TDC seedlings was performed at 4°C for 6 weeks under long-day conditions (16 h light).

Comparison of the cDNA sequences of the VRN-A1, VRN-B1, and VRN-D1 coding regions revealed the presence of polymorphic restriction sites that were used to differentiate the transcripts from the three genomes. Within the 313-bp region amplified by conserved primers Ex4-5_F1 (TCAGATCCAGC–27 and Ex8_R1 (TTGATGTGGCT[A/C]ACCATCCA), a HindIII site was present in the VRN-B1 and VRN-D1 genes but not in the VRN-A1 gene. In addition, an AluI site was present in the VRN-D1 genes but not in the VRN-A1 and VRN-B1 genes.

Forward primer Ex4-5_F1 was designed over the junction between exons 4 and 5 to avoid genomic DNA amplification. This region is conserved among the A, B, and D genomes. Reverse degenerate primer Ex8_R1 consisted of...
a mixture of two primers that differed in a single base at position 12 ("A" for the VRN-B1 gene and "C" for the VRN-A1 and VRN-D1 genes). PCR conditions included a 5° denaturation step at 94°C, followed by six cycles of touch down with annealing temperatures ranging from 58°C to 55°C in 0.5°C steps, and the 35 additional cycles at 55°C. Every cycle included a denaturation step at 94°C, the described annealing step, and an extension step at 72°C. Each step was 45 s long. Five different plants were used for each time point.

RNA samples were also collected from the leaves of diploid wheat winter accession G3116 (PI 427992, TDB, and TDE). The quantification of the accession G3116 (PI 427992, Vrn-Am1), spring accession PI 266844 (VRN-A1†), and F3 heterozygous plants from the cross between these two accessions. The VRN-A1† promoter region from PI 266844 has a 1-bp deletion in a putative CArG box that is absent in G3116 (Dubcovsky and Yan, 2003; Yan et al., 2004b). These two accessions also differ in a single nucleotide polymorphism in exon 5 that was used to identify the transcripts from the dominant VRN-A1† and recessive vrn-A1† alleles in the heterozygous plants. This polymorphism was also used to develop a cleaved amplified polymorphic sequence marker to select heterozygous F2 plants. The polymorphic region (277 bp) was first amplified with primers Tm_Ex4_F (TCTCAGGAGAGACTCTTGAA) and Tm_Ex7_R (GGAGATCCCTCCTACGAT) and then digested with restriction enzyme HinfI. The PCR amplification consisted of a 5-min denaturation step at 94°C, followed by 39 cycles, each consisting of a denaturation step at 94°C, an annealing step at 55°C, and an extension step at 72°C. Each step was 30 s long. Restriction enzyme HinfI digested the PCR amplification products into four fragments for the dominant vrn-A1† allele (PI 266844: 8 + 20 + 85 + 164 bp), but only three fragments in the recessive vrn-A1† allele (G3116: 8 + 20 + 249 bp).

Quantitative PCR

To quantify the RNA levels of each of the three VRN-1 genes, we designed three SYBR GREEN quantitative PCR systems. Forward primer SYBER_BD_F (CAGCCCTCAAACAGGTCTTCT) was designed to preferentially amplify the VRN-B1 and VRN-D1 genes, whereas forward primer SYBER_A_F (CAGCCCTAAAACAGCTTCTCA) was designed to preferentially amplify VRN-A1. Both primers were within exon 7. Reverse primers SYBER_B_R (CTCGCTGCCCTCTCCTGCA) and SYBER_D_R (CTCCCTCTCTCCTGGCTC) were designed to preferentially amplify the VRN-A1, VRN-B1, and VRN-D1 transcripts, respectively. The underlined bases in the primer sequences represent the selective polymorphic bases. The CDNA samples were checked for genomic DNA contamination by running the quantitative PCR products from the three primer pairs in agarose gels. The genomic region between these primers includes intron seven (165–167 bp) facilitating the differentiation of the CDNA and genomic DNA amplification products. We tested the genome specificity of the three SYBR GREEN quantitative PCR systems using DNA from VRN-A1, VRN-B1, and VRN-D1 BACS. In addition, we tested their amplification efficiency using six 2-fold dilutions (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32) in triplicate. These values were used to calculate the standard curves and slopes and to compare the amplification efficiency of VRN-A1, VRN-B1, and VRN-D1 relative to ACTIN. The slopes of the regression lines between the dilutions and the difference between VRN-1 and ACTIN-Cv values were all smaller than 0.1. The comparison of the VRN-1 and VRN-2 transcript levels among lines carrying different VRN-1 alleles was performed in RNA samples from the first leaves of unvernalized plants grown under long-day conditions (16 h of light). In the diploid wheat F2 experiment, the comparison among the homozygous and heterozygous classes was done using TaqMan systems developed before (Yan et al., 2003, 2004b). In the Triple Dirk experiment, we used two SYBR GREEN quantitative PCR systems to measure VRN-1 and VRN-2 in TDD, TDB, and TDE. The quantification of the VRN-1 transcripts was done by a combination of the conserved primers Ex5_F1 and Ex8_R1 described above. The quantification of the VRN-A2 transcript levels was done with primers ZCCT-A1-F (GACCAGTCTCAGCTTGAA) and ZCCT-A1-R (TTCCTCCATGCTAATAGTCCGTT). 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 homeoealleles present in hexaploid wheat.

Quantitative PCR experiments were performed in an ABI7700 using the three SYBR GREEN systems described above and ACTIN as endogenous controls (Yan et al., 2003). The 2−ΔΔCt method (Livak and Schmittgen, 2001) was used to normalize and calibrate the VRN-1 Ct 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−ΔΔCt values to correct the lack of homogeneity of variances in the untransformed data detected by Levene’s test (SAS Institute, 2003). The values presented on Figure 3 are means and SEs of the untransformed values. All statistical analyses were performed using SAS software, version 8 (SAS Institute, Cary, NC).

RNAi Transgenic Plants

The RNAi construct was made in the binary vector pMCG161 supplied by Professor Vicki Chandler. This vector contains a cassette designed for making inverted repeat transcripts of a gene, flanking a loop, which should efficiently produce a double-stranded RNA. Expression of the transgene is driven by the 35S promoter followed by the Adh1 intron. We cloned a 294-bp segment from VRN-A1† in the sense orientation between restriction sites AscI and ApaI and in antisense orientation between restriction sites Sphi and SsrI. The cloned region included the last 85 bases from exon 7, the complete exon 8, and the first 164 bp of the 5′-untranslated region. We excluded the MADS box and K-box domains from the cloned region to avoid interference with other MADS box genes. The engineered plasmid was cotransformed with UB:BAR (2:1 molar ratio) into immature embryos of spring common wheat Bobwhite, by microprojectile bombardment as described before (Okubara et al., 2002). We added 3 mg/L bialaphos to shoot regeneration and rooting media to select the transplants.

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) and by Southern blot using a probe for the 35S promoter. Transcription of the transgene in the selected T1 plants and positive T2 progeny was confirmed by RT-PCR using primers OCS-PolyA_F and Ri_AntiS_R for the transcribed region of the OCTOPINE SYNTHETASE PolyA region of the pMCG161 vector (Yan et al., 2004b). Transcription levels of the endogenous VRN-1 were investigated using a SYBR GREEN quantitative PCR system using conserved primers API_Ex3-F (CGGAAACCTGGTGTCACCATAA) and API-Ex4_R2 (TGTGTCAGTCCTCGCTT).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining such permission will be the responsibility of the requester.

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