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Authors

Pidal, Bárbara
Yan, Liuling
Fu, Daolin
et al.

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The CArG-Box Located Upstream from the Transcriptional Start of Wheat Vernalization Gene *VRN1* Is Not Necessary for the Vernalization Response

Bárbara Pidal, Liuling Yan, Daolin Fu, Fengqiu Zhang, Gabriela Tranquilli, and Jorge Dubcovsky

From the Instituto de Recursos Biológicos, INTA, Villa Udaondo, (1686) Hurlingham, Buenos Aires, Argentina (Pidal and Tranquilli); the Department of Plant Sciences, University of California, Davis, CA (Fu and Dubcovsky); and the Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, OK (Yan and Zhang). D. Fu is now at the National Key Laboratory of Crop Biology, College of Agronomy, Shandong Agricultural University, Tai'an, Shandong, China.

Address correspondence to J. Dubcovsky at the address above, or e-mail: jdubcovsky@ucdavis.edu.

Abstract

In diploid wheat (*Triticum monococcum*), and likely in other Triticeae species, the *VRN1* gene is essential for the initiation of the reproductive phase, and therefore, a detailed characterization of its regulatory regions is required to understand this process. A CArG-box (MADS-box–binding site) identified in the *VRN1* promoter upstream from the transcription initiation site has been proposed as a critical regulatory element for the vernalization response. This hypothesis was supported by the genetic linkage between CArG-box natural deletions and dominant *Vrn1* alleles for spring growth habit and by physical interactions with VRT2, a MADS-box protein proposed as a putative flowering repressor regulated by vernalization. Here, we describe a *T. monococcum* accession with a strong vernalization requirement and a 48-bp deletion encompassing the CArG-box in the *VRN1* promoter. Genetic analyses of 2 segregating populations confirmed that this *VRN1* allele is completely linked with a strong winter growth habit (*vrn-A^m1b*). Transcript levels of the *VRN1* allele with the 48-bp deletion were very low in unvernalized plants and increased during vernalization to levels similar to those detected in other wild-type *vrn-A^m1* alleles. Taken together, these results indicate that the CArG-box found upstream of the *VRN1* transcription initiation site is not essential for the vernalization response.

Key words: *CArG-box, flowering, regulatory regions, Triticum monococcum, vernalization, wheat*

Vernalization is defined as the acceleration of flowering by long-term exposures to cold temperatures and is important to protect sensitive floral meristems from freezing temperatures. Wheat (*Triticum* sp.), barley (*Hordeum vulgare* L.), and other fall-planted temperate grasses show extensive genetic differences in their response to vernalization that divides them into winter and spring forms. Varieties with winter growth habit show a strong acceleration of flowering by vernalization, whereas flowering time in varieties with a spring growth habit is less affected by vernalization. These differences are agronomically important because spring and winter varieties differ in their optimum sowing dates and are adapted to different regions (Zhang et al. 2008).

Most of the natural variation in vernalization requirement in wheat and barley is determined by vernalization genes *VRN1*, *VRN2,* and *VRN3* (McIntosh et al. 2008). These genes show significant epistatic interactions suggesting that they are part of the same regulatory pathway (Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000; Dubcovsky et al. 2005; Koti et al. 2006; Yan et al. 2006; Szũcs et al. 2007; Hemming et al. 2008). *VRN1* is a promoter of flowering (Law et al. 1975; Dubcovsky et al. 1998) that encodes a MADS-box transcription factor with high homology to the *Arabidopsis thaliana* meristem identity gene *APETALA 1* (*AP1*) (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003). The wild-type allele of *VRN1*

confers winter growth habit, and natural mutations in its promoter or first intron are associated with dominant *Vrn1* alleles that confer spring growth habit in both wheat and barley (Yan, Helguera et al. 2004; Fu et al. 2005; von Zitzewitz et al. 2005; Cockram et al. 2007). Interestingly, radiation mutants of diploid wheat (*Triticum monococcum* L., genome A^mA^m, $2n = 14$) with deleted *VRN1* genes cannot flower under any condition suggesting that *VRN1* is essential for flowering, at least in this species (Shitsukawa et al. 2007).

During the long days of spring, *VRN1* is upregulated by *VRN3* (Figure 1), a promoter of flowering (Takahashi and Yasuda 1971; Yan et al. 2006) that encodes a RAF kinase inhibitor–like protein with high homology to *Arabidopsis* protein FLOWERING LOCUS T (FT) (Yan et al. 2006). This is a small peptide that acts as a long-distance flowering signal (florigen) (Corbesier et al. 2007; Tamaki et al. 2007). *VRN3* integrates signals from the photoperiod and vernalization pathways (Yan et al. 2006; Trevaskis, Hemming et al. 2007).

Before vernalization, *VRN3* is repressed by *VRN2* (Figure 1), a locus associated with flowering repression under long days (Takahashi and Yasuda 1971; Laurie et al. 1995; Dubcovsky et al. 1998; Distelfeld, Tranquilli et al. 2009). The *VRN2* locus includes 2 linked zinc finger-*CONSTANS, CONSTANS-LIKE, TOC1 (CCT)* domain transcription factors designated as *ZCCT1* and *ZCCT2* that are expressed in leaves and apices and are downregulated during vernalization or short days (Yan, Loukoianov et al. 2004; Dubcovsky et al. 2006; Trevaskis et al. 2006). The wild-type *Vrn2* allele confers winter growth habit, and natural deletions or mutations in the CCT domain (for *CONSTANS, CONSTANS-LIKE, TOC1*) of both *ZCCT1* and *ZCCT2* (recessive *vrn2* alleles) are associated with spring growth habit in both barley and wheat (Yan, Loukoianov et al. 2004; Dubcovsky et al. 2005; von Zitzewitz et al. 2005; Dubcovsky et al. 2008; Distelfeld, Tranquilli et al. 2009). It is currently not known if the *ZCCT* genes regulate *VRN1* only through the repression of *VRN3* (Figure 1) or by additional mechanisms, but so far, no interactions have been observed between the VRN2 protein and the *VRN1* regulatory regions (Li C, Dubcovsky J, unpublished data). For more detailed descriptions of the interactions among these 3 vernalization genes, see recent reviews by Trevaskis, Hemming et al. (2007) and Distelfeld, Li et al. (2009).

Because *VRN1* is required for the initiation of the reproductive phase, a detailed characterization of its regulatory regions is essential to understand flowering initiation in the temperate cereals. In *T. monococcum* accession DV92, the potential promoter region of the wild-type *vrn-A^m1* allele extends to a maximum of 2250 bp upstream from the start codon because beyond this point there is a region of at least 67 kb of uninterrupted repetitive elements (GenBank accession number AY188331). Several ACGT-core sequences in the *VRN1* promoter have been recently shown to be the target of the wheat basic leucine zipper (bZIP) protein *FLOWERING LOCUS D-LIKE 2* that interacts with VRN3 (Li and Dubcovsky 2008). Similar interactions have been described among the orthologous genes in *Arabidopsis* (Wigge et al. 2005).

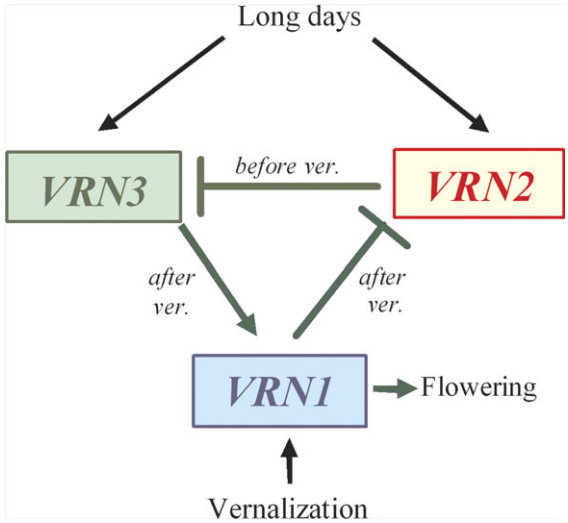

Figure 1. Hypothetical model of flowering initiation showing the interactions among *VRN1*, *VRN2*, and *VRN3* genes in a photoperiod-sensitive winter wheat. After germination in fall (relatively long days), *VRN3* is repressed by high levels of *VRN2*, precluding the induction of *VRN1*. *VRN1*, initially transcribed at very low levels in leaves and apices, is gradually upregulated during the short, cold days of winter and downregulates *VRN2*. The low levels of *VRN2* transcripts in the leaves facilitate the upregulation of *VRN3* by long days in the spring, a process mediated by photoperiod genes. VRN3 is exported to the shoot apex where it further promotes *VRN1* transcription above the threshold levels required for the induction of flowering. Arrows indicate induction, and lines ending in a crossed bar indicate repression.

An additional regulatory sequence identified in the *VRN1* promoter is a CArG-box (MADS-box–binding site) located a few base pairs upstream of the predicted site of transcription initiation (Yan et al. 2003). No additional consensus CArG-box was found in the promoter region of the *vrn-A^m1* allele (GenBank accession number AY188331). The region including this CArG-box is a binding site for the common wheat MADS-box protein VRT2 (Kane et al. 2007). The VRT2 protein is known to interact with VRN2, and when both are transformed together in tobacco (*Nicotiana benthamiana* Domin), they show stronger repression of a *VRN1* promoter::GFP reporter (Kane et al. 2007). The physical interaction between VRT2 and the *VRN1* promoter has been confirmed in *T. monococcum* (Dubcovsky et al. 2008). However, the proposed role of *VRT2* as a vernalization repressor (Kane et al. 2005) has been recently questioned by studies in barley and *T. monococcum* showing that *VRT2* and *VRN1* are both upregulated by vernalization (Trevaskis, Tadege et al. 2007; Dubcovsky et al. 2008).

A critical point for the proposed function of *VRT2* as a vernalization repressor is the role of the CArG-box to which it binds on the vernalization response. The initial suggestion that this CArG-box could play a role in

vernalization was based on the association between independent natural deletions affecting this regulatory site and spring growth habit in different accessions of *T. monococcum* (Yan et al. 2003). A 20-bp deletion in the *VRN1* promoter (*Vrn-A^{m}1a* allele, accession G2528, Figure 2) was first found to be completely linked to spring growth habit in the high-density mapping population used to positionally clone the *VRN1* gene (Yan et al. 2003). This 20-bp deletion can be considered to include or to be adjacent to the CArG-box depending on the alignment of the "AACCC" duplication present at the borders of the 20-bp deletion (Figure 2).

Two other *VRN1* alleles with deletions in the CArG-box were also found to be linked to spring growth habit (Dubcovsky et al. 2006). The first one was the *Vrn-A^{m}1f* allele found in *T. monococcum* accessions PI 503874, PI 266844, PI 393496, PI 191097, PI 191096, PI 192063, PI 2433 (Yan, Loukoianov et al. 2004), which has a 1-bp deletion within the CArG-box (Dubcovsky et al. 2006). This deletion is associated in all these accessions with the insertion of a 493-bp repetitive element in the first intron (Figure 2). Because the first intron is also important for the regulation of *VRN1* transcription (Fu et al. 2005), it is currently not possible to determine which of the altered regulatory regions in the *Vrn-A^{m}1f* allele is responsible for the spring growth habit (Dubcovsky et al. 2006). The second one was the *Vrn-A^{m}1g* allele found in accession PI 326317, which has a 34-bp deletion that truncates the CArG-box (Figure 2). This deletion is not linked with large differences in the first intron and therefore can be associated more directly to the phenotype cosegregating with this deletion (Dubcovsky et al. 2006).

A larger deletion of 48 bp, found only in the *VRN1* promoter of *T. monococcum* accession PI 355515 (Yan et al. 2003), completely eliminates the CArG-box (Figure 2). Although it was hypothesized that this deletion might be responsible for PI 355515 spring growth habit, no linkage studies have been reported so far to support this hypothesis (Yan et al. 2003). PI 355515 also carries a nonfunctional *vrn-A^{m}2* allele, which can also confer the observed spring growth habit (Yan, Loukoianov et al. 2004). We have

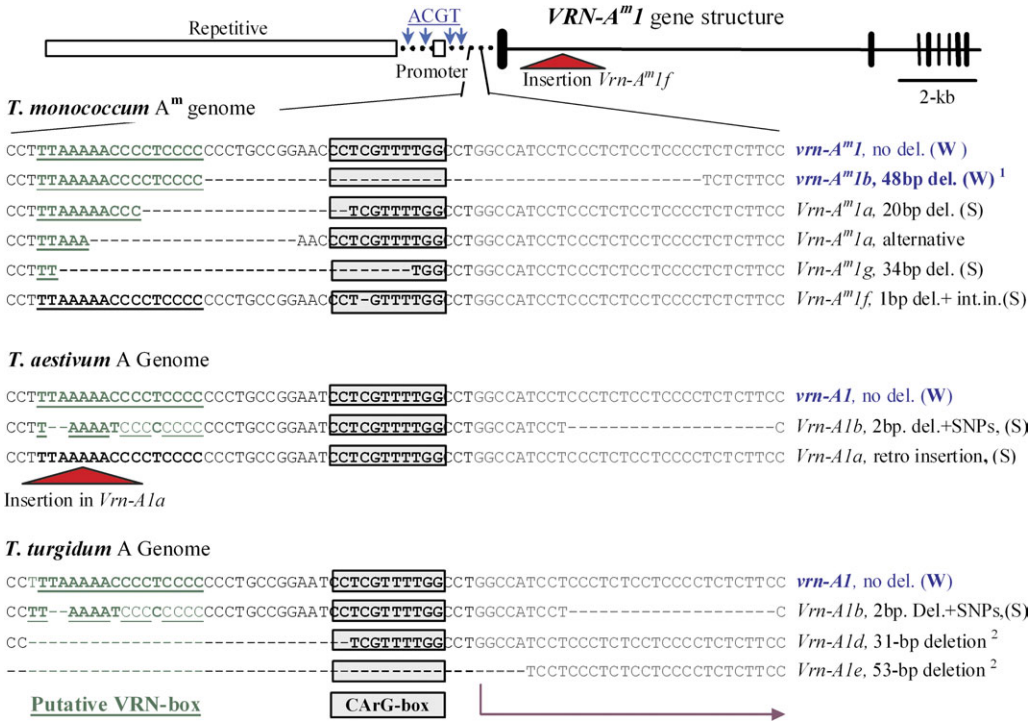

Figure 2. Top: Schematic representation of the *VRN1* gene. Exons are represented by short vertical black lines. The promoter region is indicated by dotted lines. Arrows indicate the position of ACGT-core–binding sites for FDL2. Repetitive elements in the promoter region are indicated by small and large white rectangles. The triangle indicates the positions of the insertion of a repetitive element in the first intron of the *Vrn-A^{m}1f* allele. Middle: Sequence variation at the *VRN1* promoter region immediately upstream of the transcription initiation site in the A^m genome from *Triticum monococcum* and the A genome from polyploid wheats. The putative CArG-box is indicated by bold letters and gray boxes. The insertion of a repetitive element in the promoter of the *Vrn-A1a* allele flanked by host direct duplications TTAAAAACC is represented by a triangle. The borders of all deletions are flanked by short duplications generating alternative alignments (shown only for the 20-bp deletion). Recessive (*vrn1*) and dominant (*Vrn1*) alleles are indicated on the right. S = spring growth habit, W = winter growth habit, 1 = sequence presented in this study, 2 = the *Vrn-A1d* and *Vrn-A1e* have not been studied in segregating populations yet. Bottom: Location of the putative VRN-box (underlined), the CArG-box (gray rectangles), and the transcription initiation start (arrow).

recently found another *T. monococcum* accession (PI 573525), which carries a *VRN1* allele with the same 48-bp deletion but has winter growth habit, contradicting the hypothesis that the CArG-box is critical to establish the vernalization requirement. This unexpected finding prompted us to carry out further research on 2 different populations segregating for this allele to determine the effect of the 48-bp deletion encompassing the CArG-box on the determination of the vernalization requirement in diploid wheat. We also explored the transcription profile of this allele using quantitative polymerase chain reaction (qPCR) in vernalized and unvernalized plants.

Materials and Methods

Plant Material

Triticum monococcum ssp. *monococcum* accession PI 355515 is a spring line homozygous for the 48-bp deletion in the *VRN1* promoter and also for the recessive *vrn-A^{m}2a* allele (R to W mutation at position 35 of the CCT domain), which is known to be associated with a spring growth habit (Yan, Loukoianov et al. 2004). *Triticum monococcum* ssp. *monococcum* accession PI 573525 is a line with a winter growth habit that carries a wild-type *Vrn-A^{m}2* allele and the same 48-bp deletion in the *VRN1* promoter as PI 355515. The 951-bp regions upstream of the *VRN1* start codon are identical in PI 573525 (GenBank accession number EU875080) and PI 355515 (GenBank accession number EU875079). Because winter growth habit is observed only in the presence of wild-type recessive *vrn1* alleles (Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000), the *VRN1* allele with the 48-bp deletion was tentatively designated *vrn-A^{m}1b*.

Two different F_2 populations segregating for different *VRN1* alleles in which PI 355515 was one of the parental lines were available. In the first population, PI 355515 was crossed with *T. monococcum* ssp. *monococcum* accession PI 266844, which carries dominant *Vrn-A^{m}1f* and *Vrn-A^{m}2* alleles, and 115 F_2 plants were produced and analyzed. In the second population, PI 355515 was crossed with *Triticum monococcum* ssp. *aegilopoides* accession G3116. G3116 has winter growth habit as a result of homozygous wild-type *vrn-A^{m}1* and *Vrn-A^{m}2* alleles. We evaluated 96 F_2 individuals from the PI 355515 × G3116 population and performed further progeny tests for 6 $F_{2:3}$ families homozygous for the functional *Vrn-A^{m}2* allele from G3116 and heterozygous for the *VRN1* locus. Table 1 summarizes the *VRN1* and *VRN2* genotypic constitution of the lines used in the different segregating populations.

Experimental Conditions

Plants from the 2 segregating F_2 populations were evaluated in independent experiments. In both experiments, unvernalized plants were grown in a greenhouse at 20–25 °C under 16 h of photoperiod (long day). Days to heading were registered for each line, and the experiment was terminated 60 days after the heading of the spring controls. Plants that failed to flower at this point were considered to have winter growth habit, and the termination date of the experiment was used to estimate the days to heading for the statistical analyses.

DNA Analyses

We used PCR-based markers to genotype the individual F_2 and $F_{2:3}$ plants. The *VRN1* alleles were determined using the primers for the promoter region developed by Yan et al. (2003). Primers AP1_ProDel_F1 (5′-ACAGCGGC-TATGCTCCAG-3') and AP1_ProDel_R1 (5′-TATCAGG-TGGTTGGGTGAGG-3') amplified the region flanking the 48-bp deletion. PCR amplifications were performed using an annealing temperature of 58 °C, and PCR products were analyzed in 1.5% agarose gels. The expected PCR product size for the *vrn-A^{m}1b* allele carrying the 48-bp deletion was 104 bp whereas for *Vrn-A^{m}1f* and the wild-type *vrn-A^{m}1* alleles were 151 bp and 152 bp, respectively.

VRN2 alleles were determined using CAPS primers Race3 C1N1 (5′-GCAATCATGACTATTGACACA-3') and Race C1N1 (5′-GGGCGAAGCTGGAGATGATG-3') (Yan, Loukoianov et al. 2004). After digestion of the PCR products with the restriction enzyme *Nco*I, the dominant *Vrn-A^{m}2* allele yielded a single undigested fragment of 231 bp, whereas the recessive *vrn-A^{m}2* allele was digested into 189-bp and 42-bp fragments.

qPCR Experiments

Accessions PI 355515, PI 573525 (*vrn-A^{m}1b*), and G1777 (control *vrn-A^{m}1* wild-type allele) were used for the qPCR experiments (Table 1). Unvernalized plants were grown in the greenhouse under long day conditions (16 h light), and vernalized plants were exposed to 4 °C for 6 weeks under 16 h of light. Three plants were used per genotype–treatment combination.

RNA samples were extracted from leaves using the TRIzol® method (Invitrogen, Carlsbad, CA) (Yan et al.

Table 1. Growth habit of the accessions used in this study and their alleles for *VRN1* and *VRN2*

Accession	*VRN1* allele	CArG-box	*VRN2* allele	Growth habit
PI 355515	*vrn-A^{m}1b*	48-bp Deletion	*vrn-A^{m}2a* CCT Mutation	Spring
PI 573525	*vrn-A^{m}1b*	48-bp Deletion	*Vrn-A^{m}2*	Winter
PI 266844	*Vrn-A^{m}1f*	1-bp Deletion and insertion first intron	*Vrn-A^{m}2*	Spring
G3116	*vrn-A^{m}1*	Wild type	*Vrn-A^{m}2*	Winter
G1777	*vrn-A^{m}1*	Wild type	*Vrn-A^{m}2*	Winter

2003). Both vernalized and unvernalized control plants were sampled when the vernalized plants completed 6 weeks of vernalization. Transcript levels of *T. monococcum VRN1* gene were determined using *ACTIN* as endogenous control and TaqMan systems developed before (Yan et al. 2003). All qPCR experiments were performed in an ABI PRISM 7000 SDS (Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate transcript values relative to the endogenous controls.

Results

PI 355515 × PI 266844 F_2 Segregating Population

The analysis of variance model including *VRN1*, *VRN2*, and their interaction explained 66.6% of the variation in days to heading in this experiment. This trait showed highly significant differences for both the *VRN1* ($P < 0.0001$) and *VRN2* ($P = 0.0001$) factors as well as for their interaction ($P < 0.0001$). Analysis of *VRN1* main effects (Figure 3A) showed that plants carrying the *Vrn-A^{m}1f* allele in homozygous (89 ± 4 days) or heterozygous state (82 ± 3 days) did not differ significantly from each other in days to heading ($P = 0.22$) indicating that this allele is dominant for spring growth habit. Plants homozygous for the *vrn-A^{m}1b* allele headed on average 32 days later than those homozygous or heterozygous for the *Vrn-A^{m}1f* allele, suggesting that the *vrn-A^{m}1b* allele confers winter growth habit (Figure 3A).

Analysis of the *VRN2* main effect (Figure 3B) showed that the plants homozygous for *Vrn-A^{m}2* in homozygous (107 ± 3 days) or heterozygous (102 ± 3 days) state did not differ significantly from each other in days to heading ($P = 0.55$) indicating that this allele is dominant for winter growth habit. Days to headings for the plants carrying the *Vrn-A^{m}2* allele were on average 30 days later than for the plants homozygous for the nonfunctional *vrn-A^{m}2* allele, confirming that the *VRN2* allele from PI 266844 confers winter growth habit (Figure 3B).

Because the homozygous dominant and heterozygous classes for both *VRN1* and *VRN2* showed no significant differences in days to heading, they were merged to facilitate the visualization and discussion of their interactions (Figure 3C). The 2 × 2 factorial ANOVA resulting from this merge showed, as the previous 2 × 3 analysis, highly significant differences for *VRN1*, *VRN2*, and their interactions ($P < 0.0001$). The interaction graph presented in Figure 3C shows that the differences in days to heading between *VRN1* alleles were significant only within the F_2 plants carrying at least one copy of the *Vrn-A^{m}2* allele. Conversely, differences in days to heading between the *VRN2* alleles were significant only within the F_2 plants homozygous for the *vrn-A^{m}1b* allele (Figure 3C). The only plants showing winter growth habit were those homozygous for the *vrn-A^{m}1b* allele and homozygous or heterozygous for the functional *Vrn-A^{m}2* allele. These plants flowered approximately 2 months later than the others, indicating that the *vrn-A^{m}1b* allele confers winter growth habit.

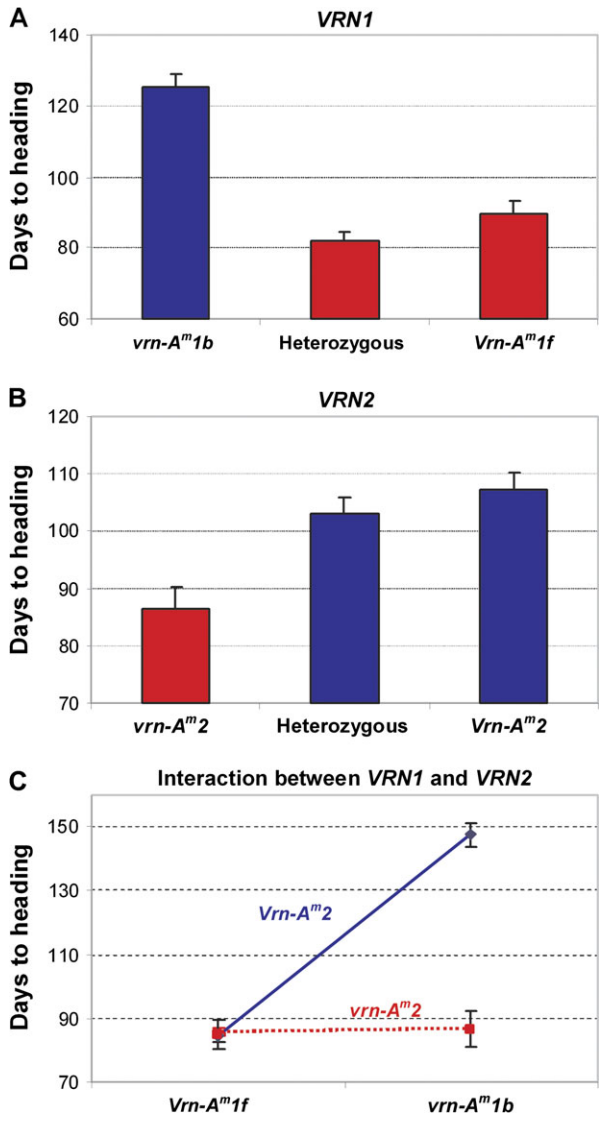

Figure 3. Segregation for heading time in the F_2 population from the cross PI 355515 (*vrn-A^{m}1b vrn-A^{m}2*) × PI 266844 (*Vrn-A^{m}1f Vrn-A^{m}2*). (**A**) Main effect of *VRN1*. *vrn-A^{m}1b*: Plants homozygous for the 48-bp deletion, *Vrn1-A^{m}f*: plants homozygous for the *Vrn-A^{m}1f* allele that confers spring growth habit. (**B**) Main effect of *VRN2*: *vrn-A^{m}2* and *Vrn-A^{m}2*: plants homozygous for the nonfunctional *vrn-A^{m}2* (spring) and functional *Vrn-A^{m}2* (winter) alleles. (**C**) Interaction graph for *VRN1* and *VRN2* allelic classes (plants homozygous for the dominant allele and heterozygous are combined). Plants homozygous for the *vrn-A^{m}1b* allele and carrying the functional *Vrn-A^{m}2* allele (homozygous or heterozygous) were the only ones with winter growth habit.

PI 355515 × G3116 F_2 Segregating Population

To compare the winter growth habit conferred by the *vrn-A^{m}1b* allele (48-bp deletion) with a previously well-characterized wild-type *vrn-A^{m}1* allele (no deletions in the promoter region), we crossed PI 355515 with the winter line

G3116 (Yan, Loukoianov et al. 2004). The F_1 plants derived from this cross, showed winter growth habit, supporting the idea that the *VRN1* allele with the 48-bp deletion is not dominant for spring growth habit.

Twenty-four individuals out of the 96 evaluated in this experiment showed spring growth habit, fitting a 3 winter:1 spring ratio ($\chi^2 = 1.00$). Analysis of the 24 spring plants with molecular markers showed that they were all homozygous for the nonfunctional *vrn-A^m2a* allele, confirming the predicted segregation for a single gene (*VRN2*) and the absence of segregation for growth habit associated to *VRN1* in this population.

To compare the effect of the *vrn-A^m1b* and *vrn-A^m1* alleles on days to heading in a winter genetic background, we selected 6 $F_{2:3}$ families homozygous for the functional *Vrn-A^m2* allele from G3116 and heterozygous for the *VRN1* locus. The 51 F_3 individuals analyzed from these 6 families all showed late heading (>150 days), which was almost 3 months later than the control plants homozygous for the *vrn-A^m2* allele (average of 44 plants = 63 ± 1 day). The 51 homozygous *Vrn-A^m2* plants were genotyped, and those carrying the different *VRN1* alleles were grouped and compared for days to heading (Table 2). Most of the plants (70.6%) failed to flower 210 days (7 months) after planting, when the experiment was finally terminated. Among the 11 plants homozygous for the *vrn-A^m1b* allele, 9 failed to flower compared with 4 among the 9 plants homozygous for the G3116 *vrn-A^m1* allele (Table 2). Among the plants that flowered before 210 days, no differences in days to heading were observed between the 2 allelic classes. Most of the heterozygous plants failed to flower, and 8 that did flower showed similar days to heading as the 2 homozygous classes (Table 2). Based on these results, we concluded that the *vrn-A^m1b* allele confers a strong winter growth habit, similar to the one observed for the G3116 *vrn-A^m1* allele.

Table 2. Days to heading for $F_{2:3}$ plants homozygous for the *Vrn-A^m2* allele and segregating for *VRN1* (PI 355515 × G3116)

VRN1 genotype	Days to heading	*N*	Days to heading	*N*
Homozygous G3116 *vrn-A^m1*	169 ± 5	5	>210	4
Heterozygous	177 ± 7	8	>210	23
Homozygous *vrn-A^m1b* (48-bp deletion)	163 ± 7	2	>210	9
Total		15		36

N indicates the number of plants within each *VRN1* allelic class.

Transcription Levels

A possible explanation for the late heading observed in plants homozygous for the 48-bp deletion (in a *Vrn-A^m2* genetic background) could be a low level of *VRN1* transcripts in these plants. To test this hypothesis, we quantified the transcription levels of *VRN1* in vernalized and unvernalized *T. monococcum* plants homozygous for the *vrn-A^m1b* allele in both homozygous *Vrn-A^m2* (PI 573525) and *vrn-A^m2* (PI 355515) genetic backgrounds (Figure 4). As a control, we included winter *T. monococcum* accession G1777, which is known to be homozygous for a recessive *vrn-A^m1* allele with a strong vernalization requirement (Yan et al. 2003).

In the plants homozygous for *vrn-A^m1b* and lacking a functional *VRN2* repressor (homozygous *vrn-A^m2*), *VRN1* was transcribed at high levels in both vernalized and unvernalized plants (Figure 4A). However, in the plants homozygous for both *vrn-A^m1b* and the functional *Vrn-A^m2* allele, *VRN1* transcript levels were significantly higher in the vernalized than in the nonvernalized plants (Figure 4B). *VRN1* transcript levels in the vernalized winter plants homozygous for the *vrn-A^m1b* allele were not significantly different ($P = 0.60$) from those in the G1777 winter control (Figure 4C). In summary, the *vrn-A^m1b* allele showed a transcription profile that did not differ substantially from the one observed in a line with a wild-type recessive *vrn-A^m1* allele (no 48-bp deletion), suggesting that this deletion is not limiting the induction of this gene by vernalization.

The higher transcript levels of *VRN1* observed in vernalized relative to unvernalized plants homozygous for the *vrn-A^m1b* allele, corresponded well with the earlier heading time observed in the vernalized plants relative to the nonvernalized ones. A 6-week vernalization treatment (4 °C, 16:8 h light:dark) of line PI 573525 (homozygous *vrn-A^m1b* and *Vrn-A^m2* alleles, winter growth habit) accelerated heading relative to the nonvernalized control by at least 41 days. The vernalized line headed 59 days after the plants were removed from the vernalization chamber, whereas the unvernalized plants remained in a vegetative state 100 days after the same reference day, when the experiment was terminated.

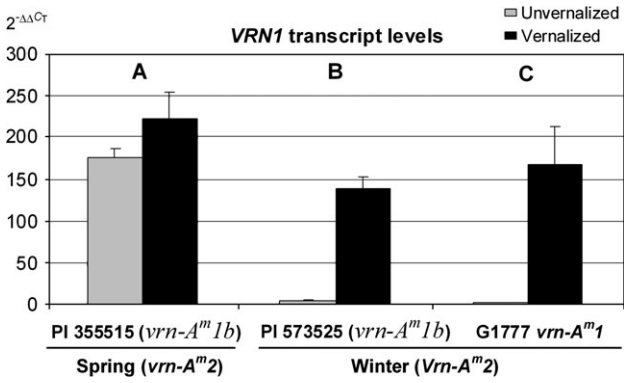

Figure 4. *VRN1* transcript levels in (**A**) PI 355515 (*vrn-A^m1b vrn-A^m2*), (**B**) PI 573525 (*vrn-A^m1b Vrn-A^m2*), and (**C**) G1777 (*vrn-A^m1 Vrn-A^m2*). Gray and black rectangles represent the averages of three unvernalized and vernalized plants, respectively. Error bars are standard errors of the means. Transcript levels were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) using *ACTIN* as endogenous control.

Sequencing of the *vrn-A^{m}1b* Allele

To test the possibility that the late heading observed in plants homozygous for the 48-bp deletion (*vrn-A^{m}1b* allele) was associated to a defective VRN1 protein, we sequenced the complete coding regions of the *vrn-A^{m}1b* allele from accession PI 355515 and deposited it in GenBank under accession number EU875079. A comparison with the available sequence of the wild-type *vrn-A^{m}1* allele from DV92 (GenBank accession number AY188331) showed 100% identity between the coding regions of the 2 alleles and therefore identical predicted proteins.

Outside the coding regions, the *vrn-A^{m}1b* allele from accession PI 355515 was also very similar to the *vrn-A^{m}1* allele from accession DV92 (GenBank accession number AY188331). Only 2 SNPs were detected between these 2 accessions among the 2500 bp sequenced from *vrn-A^{m}1b* introns (incomplete sequence). Similarly, with the exception of the 48-bp deletion, the 951-bp region upstream from the start codon of the *vrn-A^{m}1b* allele was identical to the corresponding region into the wild-type *vrn-A^{m}1* allele from accession DV92.

Discussion

In *Arabidopsis*, the transition of the stem apical meristem from vegetative to reproductive stage is redundantly regulated by the 3 paralogous genes *AP1*, *CAULIFLOWER* (*CAL*), and *FRUITFULL* (*FUL*). Plants homozygous for these 3 mutations (*ap1 cal ful*) exhibit a nonflowering phenotype in which plants continuously develop leafy shoots in place of flowers (Ferrandiz et al. 2000). The duplications that generated these 3 paralogous *Arabidopsis* genes occurred after the dicot–monocot divide and are independent of the duplications that originated the *VRN1*-related *FUL2* (= *Hv*MADS8) and *FUL3* (= *Hv*MADS3) genes in the grasses (Yan et al. 2003; Preston and Kellogg 2006). In contrast to *Arabidopsis*, the wheat *VRN1*, *FUL2*, and *FUL3* do not seem to be redundant in their ability to induce the transition of the apex to the reproductive stage because deletions of *VRN1* in *T. monococcum* are sufficient to abolish this transition (Shitsukawa et al. 2007).

Although the functional redundancy of *VRN1*, *FUL2*, and *FUL3* in grass species other than *T. monococcum* remains to be tested, Shitsukawa et al. (2007) demonstrated that *VRN1* is essential for flowering in this diploid Triticeae species. The fact that a mutation in a single gene can completely prevent flowering in *T. monococcum* emphasizes the importance of a precise characterization of the *VRN1* regulatory regions to improve our understanding of flowering initiation. Numerous environmental signals, including vernalization, photoperiod, nutrient availability, abiotic stresses, and others, have to converge and be integrated at the regulatory regions of *VRN1* to determine the initiation of the reproductive phase.

The CArG-Box Upstream from the *VRN1* Transcription Initiation Is Not Essential to Establish the Vernalization Requirement

The CArG-box located downstream of the ACGT-boxes and upstream of the transcriptional initiation site for *VRN1* (Figure 2) has been suggested as a putative regulatory site for the vernalization pathway (Yan et al. 2003). The 20-bp deletion adjacent to the CArG-box identified in *T. monococcum* accession G2528 (allele *Vrn-A^{m}1a*, Figure 2) was completely linked to spring growth habit in a very large mapping population including more than 6200 gametes (Yan et al. 2003). To rule out the possibility of linked mutations in other parts of the *VRN1* gene, we obtained the complete genomic sequence of the *VRN1* gene from G2528 (GenBank accession number AY244509) and compared it with the available sequence of the wild-type *vrn-A^{m}1* allele from DV92 (GenBank accession number AY188331). Both sequences were very similar (99.7% identical) with a single 2-bp indel within a poly-C region. The predicted proteins were identical. Based on these results, we concluded that the differences observed in the G2528 promoter are most likely the cause of the observed differences in flowering time, although we cannot rule out the possibility of additional mutations upstream or downstream of the area sequenced in this study or a potential effect of the few SNPs detected in the first intron.

Sequencing of additional *VRN1* alleles from several accessions of cultivated *T. monococcum* (Yan et al. 2003; Dubcovsky et al. 2006) revealed the presence of 1-bp, 34-bp, and 48-bp deletions (Figure 2) involving the CArG-box (Yan et al. 2003). Genetic analyses of 2 F_2 populations segregating for the 1-bp deletion and the 34-bp deletion, respectively, confirmed the linkage between these *VRN1* alleles and spring growth habit (Dubcovsky et al. 2006). As indicated before, all the accessions carrying the 1-bp deletion in the CArG-box also have the 493-bp insertion of a repetitive element in the first intron, complicating the interpretation of the effect of these 2 mutations. However, the *Vrn-A^{m}1h* allele (PI 306540, GenBank accession number DQ146423), which has an almost identical insertion at the same position of the first intron as the *Vrn-A^{m}1f* allele (GenBank accession number DQ146421) and no deletions in the CArG-box (Dubcovsky et al. 2006), is also completely linked to spring growth habit, suggesting that the first intron mutation is likely sufficient to determine spring growth habit. The *Vrn-A^{m}1g* allele (GenBank accession number DQ146422) does not have large indels in the first intron suggesting that the spring growth habit linked to this allele is likely determined by the 34-bp deletion in the *VRN1* promoter (Figure 2).

In spite of the previous results, which suggest that the CArG-box was a good candidate regulatory site for the vernalization response, the winter growth habit of PI 573525 and the F_1 plants from the cross between PI 355515 and G3116 provided strong evidence against this hypothesis. The genetic studies presented here confirmed that the complete absence of the CArG-box in the *vrn-A^{m}1b* allele is

not associated with spring growth habit. Both genetic and expression experiments confirmed that the *vrn-A^{m}1b* allele confers winter growth habit, similarly to the wild-type recessive *vrn-A^{m}1* allele. In fact, the winter growth habit determined by this allele is as strong as the winter growth habit conferred by the wild-type *vrn-A^{m}1* allele from *T. monococcum* line G3116. Most of the unvernalized plants carrying the *vrn-A^{m}1b* allele failed to flower even 7 months after planting, when a functional *Vrn-A^{m}2* allele was present.

The late flowering time of the plants homozygous for the *vrn-A^{m}1b* allele is not due to amino acid changes in the VRN1 protein because the protein coded by this allele is identical to the ones coded by the wild-type *vrn-A^{m}1* allele. In addition, the differences in flowering time are not due to low transcript levels of the *vrn-A^{m}1b* allele because qPCR studies showed transcript levels and induction profiles similar to the ones observed in plants carrying the wild-type *vrn-A^{m}1* allele. Taken together, these results indicate that the CArG-box is not essential for the vernalization response.

An Alternative Regulatory Site for Vernalization in the *VRN1* Promoter

If the CArG-box is not essential for the determination of the vernalization requirement, which regulatory region is responsible for the spring growth habit of the *T. monococcum* lines carrying the *Vrn-A^{m}1a* (20-bp deletion) and *Vrn-A^{m}1g* (34-bp deletion) alleles?

None of these alleles have large indels in the first intron or other particularly distinctive mutations in the coding regions (Dubcovsky et al. 2006), but they both share a 20-bp deletion region upstream of the CArG-box. The region upstream of the CArG-box is also altered in 2 of the dominant spring alleles found in hexaploid wheat (*Triticum aestivum* L.) (Figure 2). The *Vrn-A1a* allele, the most frequent one in spring-planted common wheat varieties (Yan, Helguera et al. 2004; Zhang et al. 2008), has an insertion of a 222-bp repetitive element 20 bp upstream of the CArG-box flanked by TTAAAAACC host direct duplications (Figure 2). The region including this repetitive element is duplicated in the *Vrn-A1a* allele (Yan, Helguera et al. 2004). The *Vrn-A1b* allele, detected in both hexaploid and tetraploid (*Triticum turgidum* L.) wheat accessions, shows a 2-bp deletion and 2 SNPs (Figure 2) in the same region affected by the retroelement insertion in the *Vrn-A1a* allele. This region partially overlaps with the deletions in the *T. monococcum Vrn-A^{m}1a* and *Vrn-A^{m}1g* alleles.

Based on the independent mutations found in the sequence upstream of the 48-bp deletion in lines known to have dominant *VRN1* alleles (TTAAAAACCCCTCCCC region, Figure 2), it is tempting to speculate that this region might be involved in the determination of the vernalization requirement. To facilitate future discussions about this region, we propose to designate it as a putative VRN-box. Although the beginning of this sequence shows good similarity to a consensus TATA-box motif, its partial deletion in the *Vrn-A^{m}1g* allele does not affect *VRN1* transcription levels relative to other *VRN1* alleles (Dubcovsky et al. 2006) suggesting the existence of alternative TATA-box or functionally equivalent motifs.

Additional *VRN1* alleles have been reported in tetraploid wheat with deletions that also affect the promoter region upstream of the CArG-box. The *Vrn-A1d* allele found in *Triticum turgidum* ssp. *dicoccoides* carries a 32-bp deletion, whereas the *Vrn-A1e* allele found in *Triticum turgidum* ssp. *dicoccum* carries a 54-bp deletion (Yan, Helguera et al. 2004). Both deletions affect the promoter region upstream of the CArG-box described above (Figure 2). Genetic studies including these lines would be important to test if the VRN-box plays a role in the regulation of vernalization requirement and, if confirmed, to better delimit this regulatory region.

Interactions between VRT2 Protein and the CArG-Box

Wheat VRT2 is an SVP-like MADS-box protein that can bind in vitro and in vivo to the CArG-box in the *VRN1* promoter (Kane et al. 2007; Dubcovsky et al. 2008). The hypothesis suggesting that the CArG-box could play an important role in the regulation of the vernalization response (Yan et al. 2003) was used by Kane et al. (2007) as an argument to propose that VRT2 is a vernalization-regulated repressor of *VRN1* (Kane et al. 2005; Kane et al. 2007). However, because the CArG-box does not appear to be essential for the vernalization response, the physical interaction between VRT2 and this regulatory region can no longer be used as an argument to support the role of VRT2 as a vernalization-regulated *VRN1* repressor. This agrees with recent *VRT2* expression results found in barley (Trevaskis, Tadege et al. 2007) and *T. monococcum* (Dubcovsky et al. 2008) showing upregulation of *VRT2* during the initial weeks of vernalization. These expression data suggest that *VRT2* is unlikely to play an important role as a vernalization-regulated repressor of *VRN1* in these species because *VRN1* is gradually upregulated after a few weeks at low temperatures in these species (Trevaskis et al. 2003; Yan et al. 2003). These results do not rule out a potential role of VRT2 as a repressor of *VRN1* that acts independently of vernalization.

It is also possible that the observed interaction between VRT2 and the CArG-box in the *VRN1* promoter plays a role later in the regulation of flower development. In *Arabidopsis*, AP1 (the homologue of VRN1) interacts with other MADS-box proteins to confer sepal and petal identity after its initial role in shoot meristem identity (Mandel et al. 1992). In agreement with this hypothesis, ectopic expression of 2 *SVP*-like genes *BM1* and *BM10* related to *HvVRT2* caused floral reversion phenotypes rather than changes in flowering time in barley (Trevaskis, Tadege et al. 2007). 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 (Trevaskis, Tadege et al. 2007). However, we have not observed unusual characteristics in the spikes of PI 355515 (*vrn-A^{m}1b* homozygous). Although this could indicate that the previous hypothesis is not correct, it is also

possible that the effect of the deletion of the CArG-box in *VRN1* is partially compensated by redundant roles of *FUL2* and *FUL3* in flower meristem identity. Transgenic or mutant plants for *VRT2* will be required to conclusively determine the function of this gene.

Epistatic Interactions between *VRN1* and *VRN2*

In addition to the regulatory elements in the promoter, genetic studies have shown that additional regulatory regions are present in *VRN1* first intron. Large deletions or insertions of repetitive elements in *VRN1* first intron correlate well with a dominant spring growth habit in polyploid (tetraploid and hexaploid) (Fu et al. 2005) and diploid wheat (Dubcovsky et al. 2006), as well as in barley (Fu et al. 2005; von Zitzewitz et al. 2005; Cockram et al. 2007).

The presence of large deletions in barley *VRN-H1* first intron has been shown to eliminate the effect of allelic variation in *VRN2* on days to heading (Takahashi and Yasuda 1971; Dubcovsky et al. 2005; Hemming et al. 2008). This differs from the effect of the 20-bp deletion in the *VRN1* promoter (*Vrn-A^{m}1a* allele, Figure 2), which reduced but did not eliminate the effect of *VRN2* allelic variation on days to heading in the cross between *T. monococcum* accessions DV92 × G2528 (Tranquilli and Dubcovsky 2000). The simultaneous segregation for *VRN1* and *VRN2* in the PI 355515 × PI 266844 population provided the opportunity to reexamine the epistatic interactions between these genes in a cross involving a different *VRN1* allele. The *Vrn-A^{m}1f* allele from PI 266844 has both mutations in the CArG-box and a repetitive element insertion in the first intron.

The repetitive element insertion in the *Vrn-A^{m}1f* allele is located upstream from the proposed critical region of the *VRN1* first intron as defined by independent overlapping deletions (Fu et al. 2005). However, it has been shown in *Arabidopsis* that the insertion of a repetitive element can affect regulatory regions adjacent to the insertion point. A repetitive element insertion in the first intron of some *FLC* alleles has been shown to originate repressive chromatin modifications in adjacent regions mediated by short interfering RNAs (Liu et al. 2004). This result suggests that the repetitive element inserted in the first intron of the *Vrn-A^{m}1f* allele may affect adjacent regulatory regions in this allele.

In the PI 355515 × PI 266844 population, the presence of the dominant *Vrn-A^{m}1f* allele suppressed the effect of the allelic variation in *VRN2* (Figure 3C), a result that has been also observed in barley *Vrn-H1* alleles with large deletions in the first intron (Dubcovsky et al. 2005). It is unlikely that this suppression is generated by the 1-bp deletion present within the CArG-box in the *Vrn-A^{m}1f* allele because the complete deletion of the CArG-box in PI 355515 has no effect on its vernalization response. Therefore, the most likely explanation for the observed epistatic interactions is that the insertion of the repetitive element in the first intron eliminated the vernalization requirement, resulting in the early induction of *VRN1* independently of the *VRN2* allele. This would generate an equivalent effect as the deletion of the first intron in the *Vrn-H1* allele used in the epistatic studies in barley, which showed similar epistatic interactions (Dubcovsky et al. 2005).

The epistatic results presented here suggest that modification or elimination of regulatory regions located within the *VRN1* first intron may have a stronger effect on the *VRN2*-mediated vernalization response than the sites located upstream of the CArG-box in the *VRN1* promoter. However, it is also possible that the smaller epistatic effect of the 20-bp deletion found in the *Vrn-A^{m}1a* allele (accession G2528) may be related to the region affected by this deletion and that longer deletions such as those observed in the *Vrn-A^{m}1g*, *Vrn-A1d*, and *Vrn-A1e* alleles that affect several more base pairs upstream of the 20-bp deletion may have a stronger epistatic effect on *VRN2*. Additional epistatic studies involving these 3 *VRN1* alleles will be necessary to distinguish between these 2 alternative explanations.

VRN1 deposited sequences

Triticum monococcum G2528: GenBank accession number AY244509 (expanded), *T. monococcum* PI 355515: GenBank accession number EU875079, *T. monococcum* PI 573525: GenBank accession number EU875080.

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