Genetic and Molecular Characterization of the *VRN2* Loci in Tetraploid Wheat[1][W][OA]

Assaf Distelfeld, Gabriela Tranquilli, Chengxia Li, Liuling Yan, and Jorge Dubcovsky*

Department of Plant Sciences, University of California, Davis, California 95616–8515 (A.D., C.L., J.D.); Instituto de Recursos Biológicos, Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina (G.T.); and Department of Plant and Soil Sciences, Oklahoma State University, Stillwater, Oklahoma 74078 (L.Y.)

Winter wheat (*Triticum* spp.) varieties require long exposures to low temperatures to flower, a process called vernalization. The *VRN2* locus includes two completely linked zinc finger-CCT domain genes (*ZCCT1* and *ZCCT2*) that act as flowering repressors down-regulated during vernalization. Deletions or mutations in these two genes result in the elimination of the vernalization requirement in diploid wheat (*Triticum monococcum*). However, natural allelic variation in these genes has not been described so far in polyploid wheat (tetraploid *Triticum turgidum* and hexaploid *Triticum aestivum*). A tetraploid wheat population segregating for both *VRN-A2* and *VRN-B2* loci facilitated the characterization of different alleles. Comparisons between functional and nonfunctional alleles revealed that both *ZCCT1* and *ZCCT2* genes are able to confer vernalization requirement and that different *ZCCT* genes are functional in different genomes. ZCCT1 and ZCCT2 proteins from nonfunctional *vrn2* alleles have mutations at arginine amino acids at position 16, 35, or 39 of the CCT domain. These positions are conserved between CCT and HEME ACTIVATOR PROTEIN2 (HAP2) proteins, supporting a model in which the action of CCT domains is mediated by their interactions with HAP2/HAP3/HAP5 complexes. This study also revealed natural variation in gene copy number, including a duplication of the functional *ZCCT-B2* gene and deletions or duplications of the complete *VRN-B2* locus. Allelic variation at the *VRN-B2* locus was associated with a partially dominant effect, which suggests that variation in the number of functional *ZCCT* genes can be used to expand allelic diversity for heading time in polyploid wheat and, hopefully, improve its adaptation to different environments.

Wheat (*Triticum aestivum*) is one of the major crop species and occupies a wide range of environments from 65°N to 45°S (Lantican et al., 2005). This wide adaptability is favored by diverse growth habits, which include winter and spring forms. Winter wheats are sown in autumn and require long exposures to cold temperatures (vernalization) to accelerate flowering. The vernalization requirement prevents flower development during winter, protecting sensitive floral organs from freezing temperatures. Spring wheats are planted in the spring or in the fall (in regions with mild winters) and do not have a vernalization requirement.

The three major genes responsible for natural variation in vernalization requirement in wheat (and also in barley [*Hordeum vulgare*]) are *VRN1*, *VRN2*, and *VRN3*. *VRN1* is a homolog of the Arabidopsis (*Arabidopsis thaliana*) meristem identity gene *APETALA1*, which determines the transition between the production of leaves and flowers at the shoot apical meristem (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). Mutagenized plants of diploid wheat (*Triticum monococcum*; $2n = 14$; A^m genome similar to the A genome of polyploid wheat) with complete deletions of the *VRN1* gene fail to flower (Shitsukawa et al., 2007), indicating that *VRN1* is essential for the initiation of the reproductive phase in this species. Several natural mutations have been identified in regulatory regions of the *VRN1* promoter or first intron, which are associated with the elimination or reduction of the vernalization requirement and consequently with spring growth habit (Yan et al., 2003, 2004a; Fu et al., 2005; vonZitzewitz et al., 2005).

VRN3 is a homolog of the Arabidopsis photoperiod gene *FLOWERING LOCUS T*, and in both species this gene up-regulates *VRN1* transcription under long days (Yan et al., 2006; Hemming et al., 2008) through interactions with its promoter (Wigge et al., 2005; Li and Dubcovsky, 2008). Before vernalization, *VRN3* is down-regulated by *VRN2*, preventing winter wheats from flowering during the fall. Vernalization results in the induction of *VRN1* and the down-regulation of *VRN2* (Loukoianov et al., 2005; Trevaskis et al., 2006), thereby releasing *VRN3* to further induce *VRN1* and initiate the reproductive phase during the long days of spring (reviewed by Trevaskis et al., 2007; Distelfeld et al.,

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* Corresponding author; e-mail jdubcovsky@ucdavis.edu.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) are: Assaf Distelfeld (adistel@ucdavis.edu) and Jorge Dubcovsky (jdubcovsky@ucdavis.edu).

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2009). The focus of this paper is the natural variation in *VRN2*.

In diploid wheat, the *VRN2* locus includes two tandemly duplicated genes designated *ZCCT1* and *ZCCT2* (Yan et al., 2004b). These genes code for proteins that are 76% identical, each including a putative zinc finger and a CCT domain (for CONSTANS [CO], CONSTANS-LIKE [CO-like], and TIMING OF CAB EXPRESSION1 [TOC1]). The 43-amino acid CCT domain is present in proteins involved in photoperiod, light signaling, and circadian rhythms and is well conserved among different plant species (Griffiths et al., 2003; Yan et al., 2004b). Mutations within the CCT domain are known to alter the functions of proteins CO, TOC1, VRN2, and PPD-H1 (Wenkel et al., 2006). It has been shown recently that the CCT domain has similarities to a region of the yeast HEME ACTIVATOR PROTEIN2 (HAP2), a subunit of the HAP2/HAP3/HAP5 complex that binds to CCAAT boxes in the promoters of many eukaryotic genes and regulates their expression (Wenkel et al., 2006).

In wheat and barley accessions with winter growth habit, *ZCCT* transcripts show a progressive decrease during vernalization (under long days) that is not observed in control plants kept at nonvernalizing temperatures (Yan et al., 2004b, Trevaskis et al., 2006). Wheat and barley *ZCCT* genes are also down-regulated by short days (Dubcovsky et al., 2006; Trevaskis et al., 2006, 2007). *GHD7* (=*OsI*), the closest homologous gene in rice (*Oryza sativa*; Xue et al., 2008), is also a long-day repressor of flowering down-regulated by short days.

All diploid wheat and barley accessions with winter growth habit studied so far have at least one functional *ZCCT* gene, whereas those with spring growth habit associated with recessive *vrn2* alleles have deletions encompassing all *ZCCT* genes or carry mutations in conserved amino acids of the CCT domains (Yan et al., 2004b; Karsai et al., 2005; Cockram et al., 2007; Szücs et al., 2007). The presence of a single functional *vrn2* allele in heterozygous plants is sufficient to confer some vernalization requirement, so only the homozygous recessive *vrn2* allele results in spring growth habit (Takahashi and Yasuda, 1971; Tranquilli and Dubcovsky, 2000).

RNA interference of *ZCCT1* in hexaploid winter wheat variety Jagger (*T. aestivum*; $2n = 42$; genomes AABBDD) reduces *ZCCT1* transcript levels and accelerates flowering, suggesting that the *VRN2* locus also plays a significant role in the regulation of flowering in hexaploid wheat (Yan et al., 2004b) and likely in tetraploid wheat (*Triticum turgidum*; $2n = 28$; genomes AABB), the donor of the A and B genomes to hexaploid wheat. Tetraploid wheats are divided into three subspecies: *T. turgidum* subsp. *dicoccoides* (wild accessions with disarticulating spikes), *T. turgidum* subsp. *dicoccon* (partially domesticated, with nondisarticulating spikes and non-free-threshing grains), and *T. turgidum* subsp. *durum* (modern free-threshing varieties). These species are characterized in this study for their natural allelic variation in the *ZCCT1* and *ZCCT2* genes. The homoeologous copies of the genes and loci from the A and B genomes are identified hereafter by including the genome designation before the gene or locus number, as is customary for wheat nomenclature (e.g. *VRN-A2*, *VRN-B2*, *ZCCT-A1*, and *ZCCT-B1*).

Allelic variation for *VRN2* has not been described so far for tetraploid or hexaploid wheat, likely due to the fact that in polyploid wheat simultaneous loss-of-function mutations at all *VRN2* homoeoloci are required to confer spring growth habit. In addition, allelic variation for *VRN2* would be detected only when alleles for winter growth habit are present at all *VRN1* loci, as alleles for spring growth habit are dominant and epistatic on *VRN2*.

RESULTS

ZCCT1 and *ZCCT2* Sequences from Diploid and Tetraploid Wheat Species

The genomic region encompassing the *VRN2* locus was previously sequenced from diploid wheat *T. monococcum* (Yan et al., 2004b; accession no. AY485644) and the A genome of tetraploid wheat *T. turgidum* subsp. *durum* var. Langdon (Dubcovsky and Dvorak, 2007; EF540321). A low-coverage sequencing of bacterial artificial chromosome (BAC) 738D05 including the *VRN-B2* locus from *T. turgidum* subsp. *durum* revealed three *ZCCT* genes, designated *ZCCT-B1* (FJ173819), *ZCCT-B2a* (FJ173823), and *ZCCT-B2b* (FJ173824), based on their sequence identity with previously reported *ZCCT* genes (Fig. 1). The coding regions of *ZCCT-B2a* and *ZCCT-B2b* are 100% identical, and their intron regions differ only by a 1-bp insertion/deletion (indel), which was used to develop a marker for the deletion. The genomic regions including the *ZCCT-B2a* and *ZCCT-B2b* genes (9.7 kb) are 99.7% identical, suggesting a recent duplication.

Additional *ZCCT* genes were sequenced from winter accessions of diploid *Triticum* and *Aegilops* species with genomes similar to the A, B, and D genomes of hexaploid wheat. These species included *Triticum urartu*, the donor of the A genome (Dvorak et al., 1988); *Aegilops speltoides* (S genome), the closest extant diploid species to the B genome of tetraploid and hexaploid wheat (Dvorak and Zhang, 1990); and *Aegilops tauschii*, the donor of the D genome of hexaploid wheat (Kihara, 1944).

The sequences for *ZCCT-D1* (FJ173818) and *ZCCT-D2* (FJ173822) were obtained from *Ae. tauschii* BAC clones 2H24, 14E16, and 78I09 (Akhunov et al., 2005), whereas the *ZCCT* genes from the other diploid species were obtained directly by PCR from genomic DNA. *Ae. tauschii ZCCT-D1* and *-D2* sequences, together with *T. urartu* sequences for *ZCCT-A1* (FJ173816) and *ZCCT-A2* (FJ173820) and *Ae. speltoides* sequences for *ZCCT-S1* (FJ173817) and *ZCCT-S2* (FJ173821), were deposited in GenBank. The phylogenetic analysis grouped the predicted ZCCT protein sequences into two distinct clades (Fig. 1), one including the ZCCT1 proteins from all species and the other including the ZCCT2 proteins from all species. This suggests that the duplication that originated these two genes preceded the divergence of the diploid *Triticum* and *Aegilops* species.

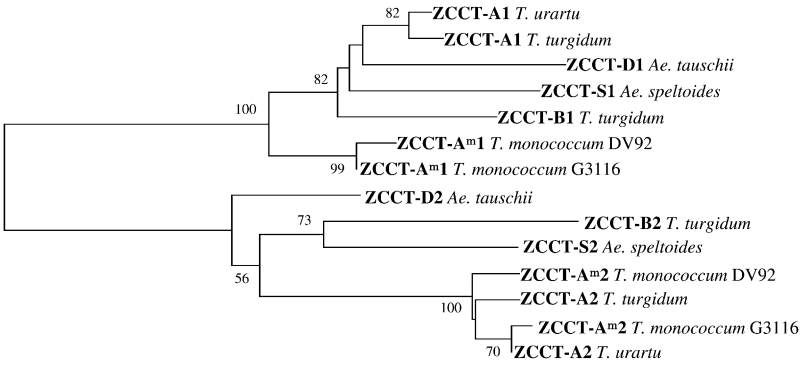

Figure 1. Neighbor-joining phylogenetic analyses of wheat ZCCT proteins. Bootstrap values based on 1,000 replications are indicated above their respective nodes. Only values above 50 are presented.

RFLP Germplasm Screen

The hybridization of Southern blots including *Dra*I-digested DNAs from wild and cultivated tetraploid and hexaploid *Triticum* accessions (see "Materials and Methods") revealed contrasting patterns of allelic diversity for different *ZCCT* genes. The shortest overlapping *Dra*I restriction fragments (767 bp from *ZCCT-A1* and 771 bp from *ZCCT-B1*) showed limited variation among accessions. Limited variation was also found for the 1,420-bp fragment corresponding to the *ZCCT-A2* gene (Fig. 2). On the contrary, the restriction fragments within the region corresponding to the *ZCCT-B2a* and *ZCCT-B2b* genes were variable in size (approximately 3–5 kb), generating multiple haplotypes (Fig. 2, lanes 1–5). Some cultivated durum lines showed two fragments in this region and others only one (Fig. 2).

The largest RFLP fragment corresponds to a third and more divergent *ZCCT* copy (*ZCCT-A3*). The *ZCCT-A3* putative coding region is only 81% to 82% identical to the other two *ZCCT* genes and has a shorter first exon that does not include the predicted zinc finger characteristic of other ZCCT proteins. So far, *ZCCT-A3* has been found only in the A genome, 16.2 kb upstream of *ZCCT-A2* (Dubcovsky and Dvorak, 2007). It is not yet known if *ZCCT-A3* is translated into a functional protein; therefore, it was not included in the allelic diversity study.

In addition to the variation in restriction fragment size, the *ZCCT-B1* and *ZCCT-B2* genes showed polymorphisms in copy number. Six *T. turgidum* subsp. *dicoccoides* accessions from Rosh Pinna, Israel, showed unusually strong hybridization signals at the restriction fragments corresponding to *ZCCT-B1* and *ZCCT-B2* (one accession is shown in Fig. 2, lane 8). The *VRN-A2* fragments from the same accessions showed no increase in hybridization intensity, confirming equal loading of DNA on the Southern blots. Based on this result, we concluded that the copy number of the *ZCCT-B1* and *ZCCT-B2* genes was amplified in the Rosh Pinna accessions.

One spring accession of *T. turgidum* subsp. *dicoccon* (PI470739), collected in the mountains of Kars, Turkey

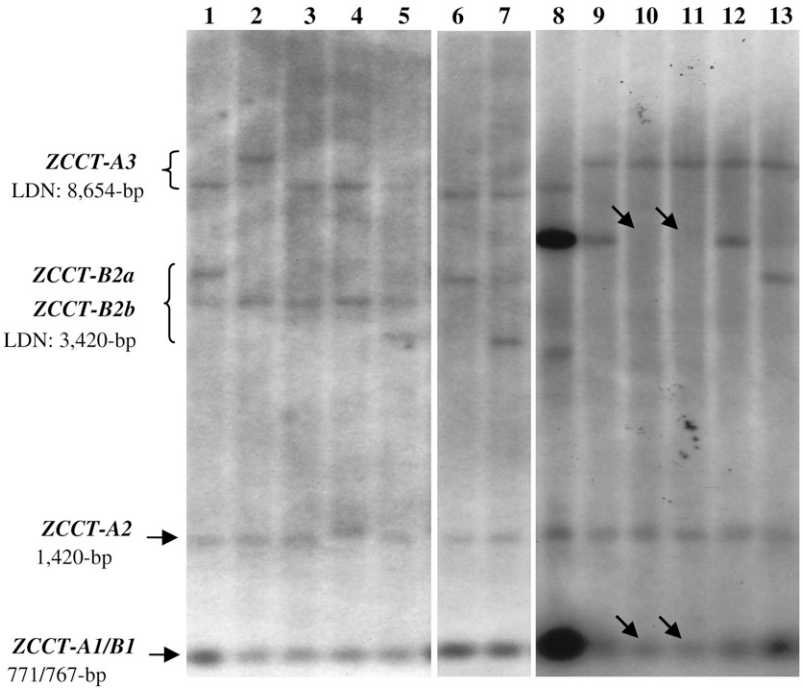

Figure 2. RFLP screening of polyploid *Triticum* accessions for variation in *ZCCT* genes. Lanes 1 to 7, *T. turgidum* subsp. *durum* accessions PI60727, PI60730, PI60731, PI60732, PI60733, PI7016, and PI10388. Lane 8, *T. turgidum* subsp. *dicoccoides* accession PI428107 (Rosh Pinna, Israel). Note the increased hybridization signal in fragments corresponding to *ZCCT-B1* and *ZCCT-B2* compared with the nonduplicated *ZCCT-A2* 1,420-bp band in the same accession. Lanes 9 to 13, *T. turgidum* subsp. *dicoccon* accessions PI470737, PI470739 (two lanes corresponding to two different plants), PI499973, and PI193881. Arrows indicate the deletion of the *ZCCT-B2* bands and the reduced intensity of the *ZCCT-A1/B1* overlapping bands. DNAs were digested with *Dra*I and hybridized with the second exon of *ZCCT-A^{m}1*.

(1,590 m above sea level), showed a deletion of the restriction fragments corresponding to the *ZCCT-B2* gene(s) and reduced hybridization intensity of the 767/771-bp fragment corresponding to *ZCCT-A1* and *ZCCT-B1* overlapping fragments (Fig. 2, lanes 10 and 11). The deletion of both *ZCCT-B1* and *ZCCT-B2* genes in PI470739 was confirmed by PCR using primers specific for *ZCCT-B1* (VRN2/B1/F3-R6) and *ZCCT-B2* (VRN2/B2/F2-R5) genes (Supplemental Table S1). These primers failed to amplify any *ZCCT* fragment from PI470739.

Molecular Characterization of the 5A-5A^m Translocation in BC_3F_2-521

The effect of the *VRN2* loci on flowering time in tetraploid wheat was studied using a plant segregating simultaneously for the *vrn-B2* deletion from PI470739 and the nonfunctional *vrn-A^{m}2* from *T. monococcum* accession DV92. The development of this plant is described in "Materials and Methods" and in Figure 3.

A total of 42 plants with winter growth habit were selected from the progeny of a BC_3F_1 line heterozygous for different vernalization genes (Fig. 3G). The winter growth habit indicates that these plants are homozygous for the recessive *vrn-A1* and *vrn-B1* alleles. Using molecular markers for *VRN-A2* (Fig. 3C) and *VRN-B2* (Fig. 3H), we selected plant 521 (BC_3F_2-521 hereafter), which was heterozygous for both *VRN-A2* and *VRN-B2* and homozygous for the recessive *vrn-A1* and *vrn-B1* alleles (Fig. 3). The progeny of this plant were used to test the effect of the different *VRN2* alleles on flowering time.

Several plants from the progeny of BC_3F_2-521 were analyzed for chromosome number and all showed 28 chromosomes, indicating that the *vrn-A^{m}2* gene was incorporated either as a complete chromosome substitution line or as a translocation line. To differentiate between these two possibilities, this line was analyzed with two molecular markers for the short and long arms of homoeologous group 5. The marker for the *PINA* gene (Bonafede et al., 2007), located in the distal region of the short arm of chromosome 5A^m and deleted in tetraploid wheat, was not present in BC_3F_2-521, confirming that *vrn-A^{m}2* was transferred to a translocated chromosome. To determine the location of the 5A-5A^m translocation, we developed a marker for the *VRN-A1* gene, which is located on the middle of the long arm. The absence of the *T. monococcum vrn-A^{m}1* allele indi-

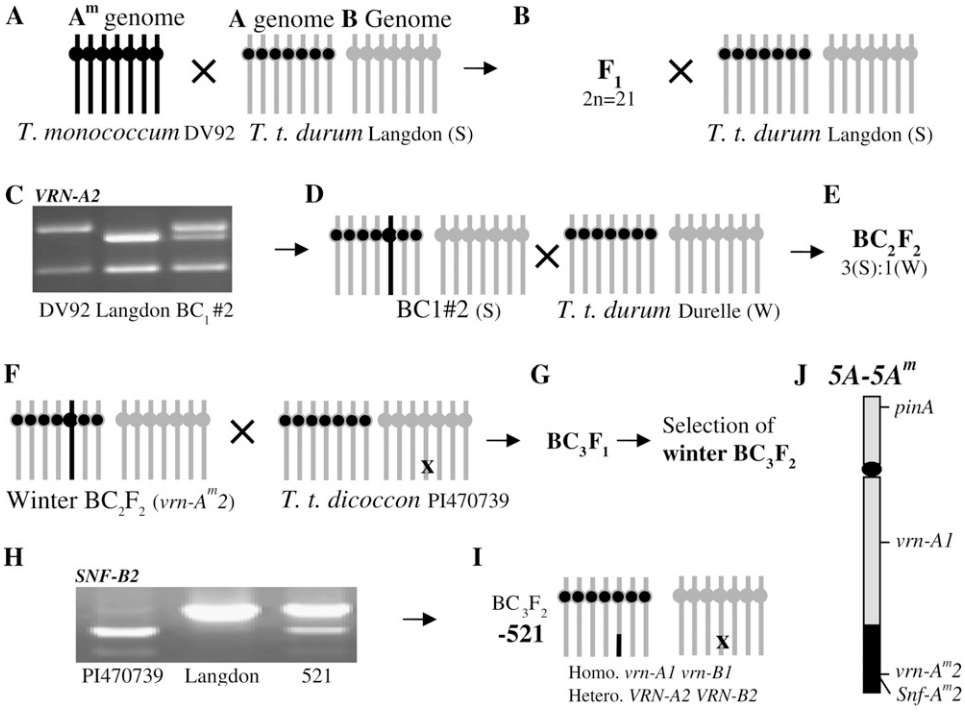

Figure 3. Generation of a tetraploid line segregating for *VRN-A2* and *VRN-B2*. A, Introgression of the nonfunctional *vrn-A^{m}2* allele from DV92 into tetraploid wheat. Black bars represent *T. monococcum* chromosomes, and gray bars represent *T. turgidum* (*T. t.*) chromosomes. B, First backcross to tetraploid wheat variety Langdon. C, Marker-assisted selection of line BC_1#2 carrying the *T. monococcum vrn-A^{m}2* allele. D, Second backcross to tetraploid winter wheat variety Durelle. E, Selection of winter growth habit BC_2F_2 plants (3:1 segregation of winter to spring). F, Backcross of a selected winter BC_2F_2 plant carrying the *vrn-A^{m}2* allele to *T. turgidum* subsp. *dicoccon* accession PI470739 carrying the *vrn-B2* deletion. G, Selection of winter BC_3F_2 plants (recessive *vrn-A1* and *vrn-B1* alleles). H, Codominant molecular marker for the *vrn-B2* deletion from PI470739 based on tightly linked gene *SNF-B2*. I, Selected line BC_3F_2-521 heterozygous for *VRN-A2* and *VRN-B2* loci. J, Graphical representation of chromosome 5A from BC_3F_2-521. The *T. monococcum* chromosome 5A^m segment carrying the *vrn-A^{m}2* allele was recombined with *T. turgidum* chromosome 5A between the *VRN-A1* and *VRN-A^{m}2* genes. S, Spring growth habit; W, winter growth habit.

cated that the recombination event occurred between the *VRN-A1* (5AL) and *VRN-A^{m}2* (5A^{m}L) loci. A marker for the deletion in the *VRN-A1* intron (Fu et al., 2005) was used to confirm that the allele present in BC_3F_2-521 was the recessive *vrn-A1* allele from Durelle, as expected from its winter growth habit. A representation of the recombined chromosome is shown in Figure 3J.

Effects of the Allelic Differences in *VRN-A2* and *VRN-B2* on Flowering Time

The nonvernalized progeny of line BC_3F_2-521 segregated into two nonoverlapping groups for flowering time. The first group included 13 early-flowering plants that headed in less than 60 d (average, 53.0 ± 0.4 d) and were classified as spring, whereas the second group included 41 late-flowering plants that took more than 90 d for heading (average, 139.1 ± 4.6 d) and were classified as winter (Fig. 4A). The group with spring growth habit was less variable than the group with winter growth habit, which showed two peaks, likely associated with the presence of homozygous and heterozygous lines (Fig. 4A). The observed ratio between spring and winter plants differed significantly from a 1:15 ratio segregation (two dominant genes; $\chi^2 = 29.3$, $P < 0.0001$) but not from a 1:3 ratio (one dominant gene; $\chi^2 = 0.025$, $P = 0.88$). The large differences in heading time observed among the nonvernalized plants disappeared when plants were vernalized (Fig. 4B).

The same plants were genotyped with *VRN-A2* and *VRN-B2* markers to determine which locus was responsible for the observed segregation in heading time (Fig. 5). No significant differences were detected among the *VRN-A2* genotypic classes (Fig. 5A), indicating that both *T. turgidum* (PI470739) and *T. monococcum* DV92 have recessive *vrn-A2* alleles. This indicates that none of the *ZCCT* genes present at the *VRN-A2* locus is able to confer a vernalization requirement.

Genotyping with the codominant *SNF-B2* marker tightly linked to the *VRN-B2* locus showed that all 13 spring plants were homozygous for the recessive *vrn-B2* allele from PI470739 (Fig. 5B), a result that was confirmed using PCR primers specific for the *ZCCT-B1* and *ZCCT-B2* genes (Supplemental Table S1). These results indicate that variation at the *VRN-B2* locus was responsible for the segregation in flowering time observed in the progeny from BC_3F_2-521.

The effect of the functional *VRN-B2* allele on heading time was partially dominant. Plants homozygous for the functional *VRN-B2* allele (Langdon/Durelle) flowered 159 ± 5.3 d after sowing, whereas those heterozygous for *VRN-B2* flowered significantly ($P < 0.001$) earlier (120 ± 4.3 d after sowing). Heading time of the heterozygous plants was only 14 d later than the midpoint between the two homozygous classes (106 d). Using these numbers, the degree of dominance was calculated to be 0.26 (14 d/53 d, with 0 = completely additive and 1 = completely dominant). This value indicates a relatively small dominant effect.

The differences in heading time between the *VRN-B2* allelic classes disappeared when the plants were vernalized. Vernalized plants carrying the functional *VRN-B2* allele headed almost at the same time (73.7 ± 1.4 d) as the vernalized plants homozygous for the *vrn-B2* deletion (74.1 ± 1.2 d; $P = 0.26$). Differences between the *VRN-A2* allelic classes were also not significant ($P = 0.13$). A two-way factorial ANOVA including vernalization and *VRN-B2* alleles as factors showed a highly significant interaction between *VRN-B2* alleles and vernalization ($P < 0.0001$), confirming that the effect of this locus on heading time was the result of differences in vernalization requirement.

Sequence Diversity of the *ZCCT1* and *ZCCT2* Genes

The *ZCCT1* and *ZCCT2* genes present in the functional *VRN-B2* and nonfunctional *VRN-A2* loci from BC_3F_2-521 were sequenced to determine if the differences in functionality were associated with specific mutations in the conserved CCT domain. For comparison, a consensus CCT sequence was generated from different classes of CO-like proteins found in plants (Griffiths et al., 2003; Yan et al., 2004b) and was represented using WebLogo (Crooks et al., 2004). The consensus sequence was aligned with the CCT domain sequences from winter accessions of wild diploid progenitors of cultivated wheat (*T. urartu*, *Ae. speltoides*, and *Ae. tauschii*; Fig. 6). The main differences among species are summarized in Table I.

ZCCT-A1

The predicted ZCCT-A1 protein corresponding to the nonfunctional *VRN-A2* locus from BC_3F_2-521 has a

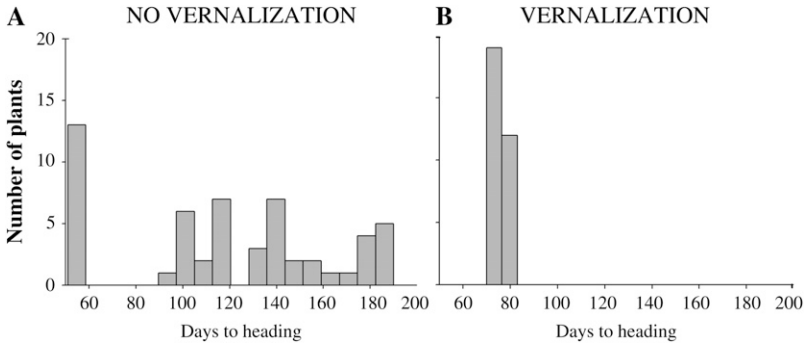

Figure 4. Segregation for heading time. Frequency distribution of days to heading for the progeny of winter plant BC_3F_2-521 (heterozygous for both *VRN2* loci). A, Unvernalized plants (1:3 segregation of spring to winter growth habit). B, Vernalized plants (no segregation for flowering time).

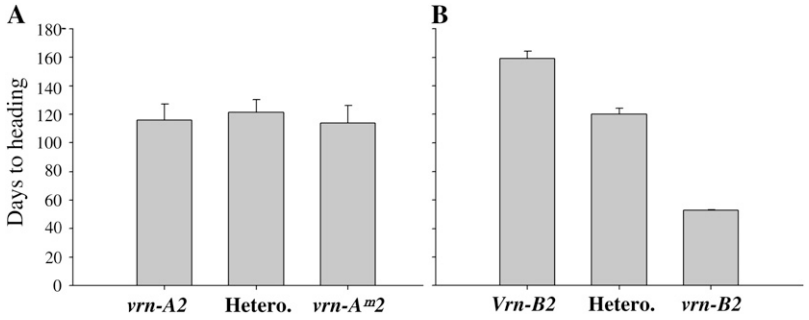

Figure 5. Effects of *VRN-A2* and *VRN-B2* alleles on heading time in nonvernalized plants. A, No significant differences in heading time were observed between lines homozygous for the *vrn-A*^{m}*2* allele from *T. monococcum* DV92 and the one from *T. turgidum* subsp. *dicoccon* PI470739 (*vrn-A2*). B, Lines homozygous for the Langdon/Durelle *VRN-B2* allele headed significantly later ($P < 0.01$) than the heterozygous or homozygous lines for the *vrn-B2* deletion (PI470739). ''Hetero.'' indicates heterozygous plants, whereas the two other classes are homozygous for the indicated alleles.

mutation from R to C at position 39 of the CCT domain (designated R39C hereafter; Fig. 6). This position of the CCT domain is well conserved among CCT domains from other CO-like proteins and HAP2 proteins (Fig. 6).

The R39C mutation was detected in all 37 cultivated *T. turgidum* subsp. *durum* accessions analyzed in this study but was polymorphic in cultivated *T. turgidum* subsp. *dicoccon* (present in 11 of 22 accessions) and wild *T. turgidum* subsp. *dicoccoides* (present in 10 of 19 accessions; Table II). One accession from Asia Minor (PI355454) showed an additional R35Q mutation. The 11 accessions of *T. turgidum* subsp. *dicoccon* that lack the R39C mutation all have the R35W mutation. The R35W mutation was also found in eight of the nine accessions of *T. turgidum* subsp. *dicoccoides* that do not have the R39C mutation. *T. turgidum* subsp. *dicoccoides* accession 10-85 collected at Ammiad in Israel was the only one with no mutations in the CCT domain from ZCCT-A1 (Table II).

In addition to the R39C mutation, the ZCCT-A1 protein has a deletion of seven amino acids relative to the ZCCT-A^{m}1 protein from *T. monococcum*. These seven amino acids are located immediately downstream of the putative zinc finger domain from amino acids 49 to 55 (numbers are relative to the initial Met in ZCCT-A^{m}1). A

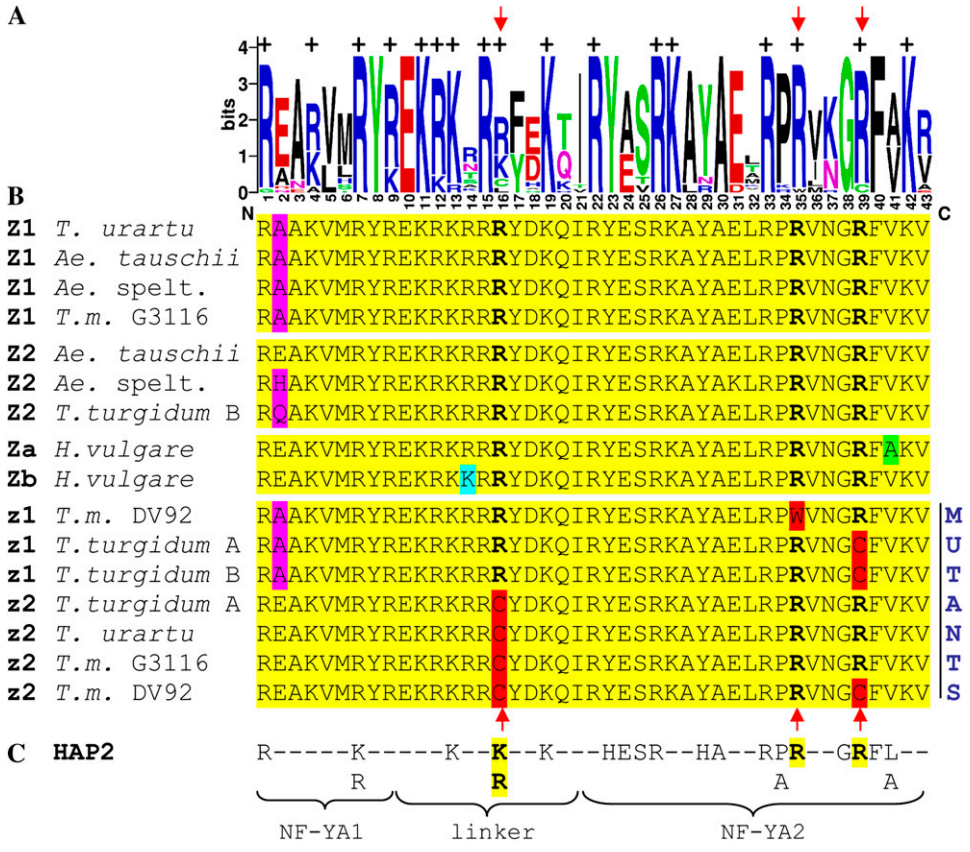

Figure 6. Alignment of the CCT domains from different plant species. A, WebLogo representation of consensus sequence from CCT domains from rice and Arabidopsis CO-like proteins (Griffiths et al., 2003; Yan et al., 2004b) and nonmutant *ZCCT1* and *ZCCT2* proteins. The sizes of the different letters are proportional to the frequency of the amino acid in the multiple sequence alignment. B, Alignment of the CCT domains from wheat and *Aegilops* ZCCT1 and ZCCT2 proteins. Arrows point to natural mutations discovered in conserved amino acids that may affect protein function. *Ae. spelt.*, *Ae. speltoides*; *T.m.*, *T. monococcum*. C, Conserved amino acids between CCT and HAP2. Subdomains NF-YA1 (interacts with HAP3 and HAP5) and NF-YA2 (interacts with CCAAT DNA sequences) are indicated below.

Table I. *Summary of polymorphisms between ZCCT1 and ZCCT2 proteins from different species and genomes*

Numbers below the protein names indicate the positions of the amino acids in the CCT domain. Del, Seven-amino acid deletion. Bold letters indicate mutated amino acids.

Genotype	VRN2 Function	ZCCT1				ZCCT2		
		Del	16	35	39	16	35	39
T. urartu	Functional[a]	Yes	R	R	R	**C**	R	R
Ae. speltoides	Functional[a]	No	R	R	R	R	R	R
Ae. tauschii	Functional[a]	No	R	R	R	R	R	R
T. monococcum 3116	Functional	No	R	R	R	**C**	R	R
T. monococcum DV92	Nonfunctional	No	R	**W**	R	**C**	R	**C**
T. turgidum 521 A genome	Nonfunctional	Yes	R	R	**C**	**C**	R	R
T. turgidum 521 B genome	Functional	No	R	R	**C**	R	R	R

[a]Based on winter growth habit but not confirmed by genetic studies.

screening using primers VRN2/22F+R (Supplemental Table S1) showed that the same deletion was present in all 78 tetraploid wheats tested in this study (Table II) and in 103 of 107 *T. urartu* accessions. The *ZCCT-A1* genes from 15 *T. urartu* accessions were fully sequenced, and none of them have mutations in the CCT domain.

ZCCT-A2

The predicted ZCCT-A2 protein corresponding to the nonfunctional *VRN-A2* locus from BC_3F_2-521 has a mutation from R to C at position 16 of the CCT domain (R16C). This position is well conserved (R or K) among CCT domains from other CO-like proteins (except for CO-like group II) and HAP2 proteins (Fig. 6).

All 48 accessions of cultivated tetraploid wheat sequenced for this gene have the R16C mutation in the CCT domain. This mutation was also found in the 15 accessions of *T. urartu* and four accessions of *T. monococcum* (ZCCT-A^m2) but was not detected in the predicted ZCCT2 proteins from *Ae. tauschii* or *Ae. speltoides* (Table I; Fig. 6). Three of the four *T. monococcum* acces-

Table II. *Summary of different ZCCT-A1 and ZCCT-B1 alleles in a collection of wild T. turgidum subsp. dicoccoides, partially domesticated T. turgidum subsp. dicoccon, and modern cultivated T. turgidum subsp. durum*

PI accessions are from the National Small Grain Collection (United States), and other numbers are population numbers from the University of Haifa. Numbers below the gene names indicate their positions within the CCT domain. All ZCCT-A1 and ZCCT-B1 proteins included here have an R at position 16. Bold letters indicate mutated amino acids.

Germplasm	Country	Species	ZCCT-A			ZCCT-B		
			A1		A2	B1		B2
			35	39	16[a]	35	39	No
10-85	Israel	*T. turgidum* subsp. *dicoccoides*	R	R	**C**	R	R	1[b]
1, 8, 17, 27, 41, PI428055	Israel, Turkey		R	**C**	**C**	R	R	1
PI428028, PI428036, PI428041, PI428066, PI428070, PI428072, PI428079, PI428082	Turkey		**W**	R	**C**	R	R	1
5, 11, 30, 42	Israel		R	**C**	**C**	R	**C**	1
PI355498, CItr17676, PI606325, PI182743, PI352329, PI94627, PI352352, PI352357, PI352367	Asia Minor, Israel, Syria, Turkey	*T. turgidum* subsp. *dicoccon*	**W**	R	**C**	R	R	1
PI319868, PI319869	Turkey		**W**	R	**C**	R	**C**	**2**
PI94640, PI254158, PI254180, PI347230, PI470737, PI470738, PI355496, CItr17675	Iran, Israel, Turkey, Lebanon		R	**C**	**C**	R	R	1
PI470739	Turkey		R	**C**	**C**	Deletion		
PI355454	Asia Minor		**Q**	**C**	**C**	R	**C**	**2**
PI352347	Israel		R	**C**	**C**	R	**C**	**2**
Adamello, Appio, Appulo, Capelli, Ciccio, Cirillo, Colosseo, Duilio, Karel, Latino, L35, Ofanto, Russello SG7, San Carlo, Saragolla, Trinakria, Valbelice, Valforte, Valnova, Varano, Vitron, WB881, Zenit, Inrat 69, Karim, Khiar, Exeldur, Durfort, Nefer, Neodur, Aconchi 89, Altar 84, Mexicali 75, Colorado, Kronos, Produra	Italy, Tunisia, France, Mexico, United States	*T. turgidum* subsp. *durum*	R	**C**	**C**	R	**C**	**2**
Messapia	Italy		R	**C**	**C**	R	**C**	1

[a]Since the R16C mutation is fixed in ZCCT-A2 and ZCCT-A^m2, we checked only one accession from each group for this mutation. [b]1 indicates that only one of the two polymorphic sites was detected but does not completely rule out the existence of the duplication.

sions (including DV92) have an R39C mutation in addition to the R16C mutation in ZCCT-A^{m}2.

ZCCT-B1

The predicted ZCCT-B1 protein corresponding to the functional *VRN-B2* locus has the same R39C mutation as the ZCCT-A1 protein coded by the nonfunctional *VRN-A2* locus (Table I; Fig. 6). The R39C mutation was conserved in the predicted ZCCT-B1 proteins from the 37 accessions of cultivated durum wheat (Table II) and was polymorphic in wild *T. turgidum* subsp. *dicoccoides* (present in four of 19 accessions) and cultivated *T. turgidum* subsp. *dicoccon* (present in four of 22 accessions). No additional changes in amino acids were detected in the CCT domain of ZCCT-B1 (Table II).

ZCCT-B2

The predicted ZCCT-B2a and ZCCT-B2b proteins corresponding to the functional *VRN-B2* locus from tetraploid variety Langdon have no mutations in any of the conserved amino acids of the CCT domain (Table I; Fig. 6). This was also the case for the other 37 cultivated durum accessions (Fig. 6).

A screening for the 1-bp indel characteristic of the *ZCCT-B2* gene duplication using PCR primers VRN2/B2/F2+R5 (Supplemental Table S1) failed to detect the duplication in *T. turgidum* subsp. *dicoccoides*. Although the detection of the two *ZCCT-B2* forms with this PCR marker is sufficient to confirm the presence of the duplication, the detection of a single sequence needs to be interpreted with caution because it can indicate either the absence of the duplication or the absence of the 1-bp indel polymorphism. The fact that all of the *T. turgidum* subsp. *dicoccoides* accessions included in the RFLP screen show a single *ZCCT-B2* fragment (with the exception of the Rosh Pinna accessions) provides additional indirect evidence for the absence of the duplication in most wild accessions.

In *T. turgidum* subsp. *dicoccon*, the presence of the *ZCCT-B2* duplication was confirmed in the four accessions that carry the R39C mutation at the *ZCCT-B1* gene (PI319868, PI319869, PI355454, and PI352347; Table II). The presence of the *ZCCT-B2* duplication was also confirmed among most of the modern *T. turgidum* subsp. *durum* varieties (36 of 37), with Messapia as the only exception (Table II). Many of the cultivated durum varieties showed two fragments in the RFLP screening (Fig. 2).

Expression of *ZCCT1* and *ZCCT2* Genes in Tetraploid Wheat

Quantification of transcript levels of *ZCCT1* and *ZCCT2* in tetraploid wheat leaves collected from 3-, 4-, and 5-week-old plants showed that the average transcript levels of *ZCCT2* were significantly higher than those of *ZCCT1* for all three time points (Fig. 7). Since quantitative reverse transcription-PCR primers (Supplemental Table S1) were designed to differentiate *ZCCT1* from *ZCCT2* but not A from B genome copies of the same gene, the transcript levels presented in Figure 7 include both A and B homoeologues for each gene.

DISCUSSION

The results presented here indicate that the differences among ZCCT proteins coded by genes corresponding to functional and nonfunctional *VRN2* alleles are concentrated in the CCT domain. This 43-amino acid domain is well conserved in CO and CO-like proteins (defined as being more similar to CO than to other Arabidopsis proteins like TOC1) from mosses, gymnosperms, and angiosperms, indicating an ancient origin (Griffiths et al., 2003). The CCT domains are involved in the nuclear localization of CO and CO-like proteins but also have additional roles. In Arabidopsis, the *co-7* CCT mutation does not alter the nuclear localization of the CO protein but delays flowering significantly (Robson et al., 2001). It is possible that some mutations in the CCT domain may limit its ability to interact with other proteins (Kurup et al., 2000).

It was shown recently that the CCT domains from Arabidopsis CO and COL15 can interact with several AtHAP3 and AtHAP5 proteins in yeast, and this interaction was confirmed in plant cells and in vitro (Ben-Naim et al., 2006; Wenkel et al., 2006). Wenkel et al. (2006) proposed that CCT proteins act by replacing the HAP2 subunit of the HAP2/HAP3/HAP5 complex, altering the ability of this complex to bind to the

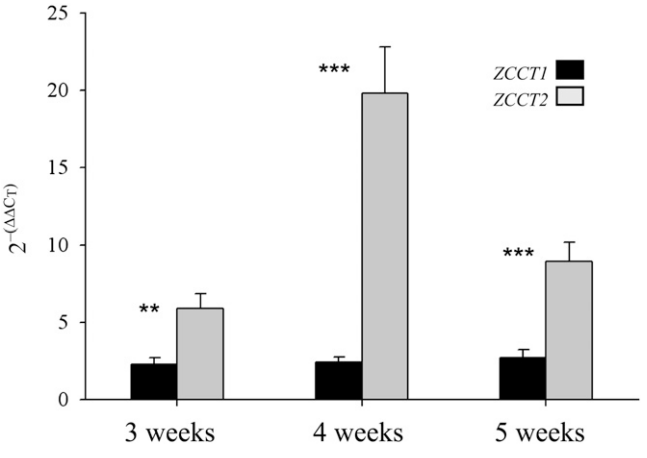

Figure 7. Expression of *ZCCT1* and *ZCCT2* in tetraploid wheat. Transcript levels of *ZCCT1* and *ZCCT2* were compared in samples from leaves of a tetraploid winter line derived from BC_3F_2-521 (homozygous for the functional alleles *VRN-A2* from *T. turgidum* subsp. *dicoccon* accession PI470739 and *VRN-B2* from varieties Langdon or Durelle). Eight plants were sampled from 3-, 4-, and 5-week-old plants. Gray bars represent *ZCCT2* transcripts, and black bars represent *ZCCT1* transcripts. Amplification primers are conserved for the A and B homoeologous copies of each gene. Asterisks indicate significant differences (*t* test): ** $P \leq 0.01$, *** $P \leq 0.001$.

CCAAT boxes in the promoters of target genes. Overexpression of *AtHAP3b* was shown to promote early flowering, probably through an interaction with CO or COL proteins, while *hap3b*, a null mutant of *HAP3b*, delayed flowering under long days but not under short days (Cai et al., 2007).

CCT domains and HAP2 proteins have similar amino acids at 18 positions, which are also well conserved within each group of proteins from mosses to vascular plants (Wenkel et al., 2006). Conservation of these amino acids for more than 400 million years suggests that they play critical roles in the proper function of these proteins. Six independent mutations at four of these conserved positions have been shown to disrupt the function of CO (Putterill et al., 1995; Robson et al., 2001), VRN2 (Yan et al., 2004b), TOC1 (Strayer et al., 2000), and PPD-H1 (Turner et al., 2005) proteins. These six mutations are all located within the CCT domain region that corresponds to the NF-YA2 subdomain of the HAP2 protein (Fig. 6). This HAP2 subdomain has been modeled and predicted to interact with the DNA of the CCAAT box (Romier et al., 2003). In addition to the NF-YA2 subdomain, the HAP2 protein has another NF-YA1 subdomain proposed to interact with the HAP3/HAP5 dimer and a linker region between these two subdomains (Romier et al., 2003). It is tempting to speculate that the ZCCT proteins may also regulate flowering time through interactions with HAP proteins and that mutations in the CCT domain may affect the ability of the ZCCT proteins to interact with DNA or with HAP3/HAP5 dimers. We recently confirmed that ZCCT proteins can interact with several wheat HAP3 and HAP5 proteins in yeast two-hybrid systems (C. Li, A. Distelfeld, and J. Dubcovsky, unpublished data), providing additional support for this hypothesis.

Interestingly, the three CCT mutations identified here in ZCCT proteins coded by genes located in nonfunctional *VRN2* loci are located at positions 16, 35, and 39, which are conserved both between and within the CCT domains and HAP2 proteins (Fig. 6). These three positions of the CCT domain are conserved in many plants, including mosses. Position 16 is either K or R (both positively charged amino acids) in Arabidopsis CO, CO-like (except those of class II), and all HAP2 proteins, whereas Arg residues at positions 35 and 39 are invariant among the same proteins (Fig. 6). All three mutations (R16C, R35W, and R39C) are associated with high negative BLOSUM62 scores (−3), which are indicative of changes involving amino acids with very different biochemical properties.

Taken together, the high negative BLOSUM62 scores and the conserved CCT/HAP2 positions where these mutations occurred suggest that these three mutations have a high probability of disrupting or altering the function of the mutant ZCCT proteins. The importance of CCT position 35 has been confirmed independently in Arabidopsis CO, as an induced ethylmethane sulfonate mutation at this position (*co-7*) produces a severe effect on flowering time (Robson et al., 2001).

The Two-ZCCT Hypothesis

Assuming that the mutations at CCT positions 16, 35, and 39 can disrupt the function of the ZCCT proteins, the following model can explain the complex results presented here. We propose that both ZCCT1 and ZCCT2 have the ability to delay flowering and confer a vernalization requirement. We will refer to this model hereafter as the ''two-ZCCT'' hypothesis to facilitate the discussion. The first corollary of this hypothesis is that the presence of a functional copy of at least one of these two genes would be sufficient to confer a vernalization requirement. The second corollary of this hypothesis is that mutations in both genes are required to completely disrupt the function of a particular *VRN2* locus. The following arguments are presented to support this hypothesis.

Similarity of ZCCT1 and ZCCT2 CCT Domains

The CCT domains from ZCCT1 and ZCCT2 are almost identical among functional alleles from different species (Fig. 6). The only difference between them is found at the second amino acid, which is fixed for A in the ZCCT1 proteins and varies between E, H, and Q in the wheat ZCCT2 proteins (and the barley ZCCT proteins). The second amino acid of the CCT domain is also variable among CO-like proteins and is not conserved with the HAP2 protein (Fig. 6), suggesting that it may not be a critical position for the function of the CCT domain. Therefore, it is reasonable to assume that ZCCT1 and ZCCT2 may have the ability to perform similar functions.

Nonfunctional vrn2 Alleles

The two-ZCCT hypothesis predicts that all recessive *vrn2* alleles would have nonfunctional mutations at both ZCCT1 and ZCCT2 proteins. In agreement with this prediction, the recessive *vrn-A2* allele from BC_3F_2-521 has the R39C mutation in ZCCT-A1 and the R16C mutation in ZCCT-A2 (Table I). The deletion of seven amino acids found downstream of the putative zinc finger in the ZCCT-A1 protein in tetraploid variety Langdon (Dubcovsky and Dvorak, 2007) and BC_3F_2-521 (from PI470739) does not seem to be critical for the function of the ZCCT-A1 protein, since a similar deletion was observed in *T. urartu* accession PI428180, which has a winter growth habit (functional *VRN-A2* allele) and a likely nonfunctional ZCCT-A2 protein (Table I).

The available information from *T. monococcum* also supports the two-ZCCT hypothesis. Cultivated *T. monococcum* accession DV92 has a recessive *vrn-A^m2* allele that is associated with the R35W mutation in ZCCT-A^m1 and both R16C and R39C mutations in ZCCT-A^m2 (Yan et al., 2004b). An additional survey of 39 spring accessions of cultivated *T. monococcum* carrying the recessive *vrn-A^m2* allele showed that all have either deletions encompassing both *ZCCT-A^m1* and *ZCCT-A^m2* genes (17 accessions)

or R35W mutations in ZCCT-A^m1 (22 accessions; Yan et al., 2004b). Although ZCCT-A^m2 was not analyzed in detail, all four accessions of *T. monococcum* for which the sequence of this gene is available have the R16C mutation in ZCCT-A^m2. Since the R16C mutation has been detected in all of the *ZCCT-A2* (*T. urartu* and *T. turgidum*) and *ZCCT-A^m2* genes sequenced so far, it is reasonable to assume that this mutation occurred before the divergence of the A and A^m genomes and, therefore, that it is likely fixed in the polyploid wheat species.

Based on the limited information available at the time of cloning *VRN-A^m2*, Yan et al. (2004b) concluded that *ZCCT-A^m1* was *VRN-2* and that *ZCCT-A^m2* was not important for the determination of the winter growth habit. At that point, it was not clear that the *ZCCT-A^m2* gene in *T. monococcum* was fixed for a nonfunctional allele, simplifying the detection of the segregation for the R35W mutation in *ZCCT-A^m1*. Although the published conclusion is valid for *T. monococcum*, our results indicate that it cannot be generalized to all Triticeae species.

Functional VRN2 Alleles

None of the 16 winter accessions of cultivated *T. monococcum* screened so far for ZCCT-A^m1 has the R35W mutation (Yan et al., 2004b). Available sequences for ZCCT-A^m2 for two of these winter accessions (AY485976 and AY485975) showed that they both have the R16C and R39C mutations, suggesting that winter growth habit in *T. monococcum* is conferred only by the ZCCT-A^m1 protein.

The same is true for the winter accessions of *T. urartu*. The 15 accessions of *T. urartu* sequenced so far all have the R16C mutation in the CCT domain of ZCCT-A2 and no mutations in ZCCT-A1 (Table I). This suggests that the winter growth habit in *T. urartu* is also conferred by ZCCT-A1.

The molecular characterization of the functional *VRN-B2* allele provided the strongest support to the two-ZCCT hypothesis. The ZCCT-B1 protein found in the parental lines of BC_3F_2-521 (Langdon/Durelle) has an R39C mutation identical to the one found in the ZCCT-A1 protein from the nonfunctional *VRN-A2* allele (Table I). The low BLOSUM62 score (−3) and the fact that this mutation alters a conserved position across HAP2 proteins and CCT domains (Fig. 6) suggest that this ZCCT-B1 protein is nonfunctional. In contrast, the ZCCT-B2 protein has no mutations in the conserved amino acids of the CCT domain. The Q mutation found in ZCCT-B2 is associated with a positive BLOSUM62 score (+2), indicative of similar biochemical properties. In addition, CCT position 2 is variable among the CCT domains of ZCCT2 and CO-like proteins and is not conserved with the HAP2 proteins (Fig. 6). These observations suggest that this mutation may not have a negative impact on the structure or function of ZCCT-B2 and that this protein rather than ZCCT-B1 is the one conferring the strong vernalization requirement observed in the late-flowering lines from the BC_3F_2-521 progeny.

Functional VRN-S2 (Ae. speltoides) and VRN-D2 (Ae. tauschii) Alleles

Most of the *Ae. speltoides* and *Ae. tauschii* accessions have a winter growth habit, which suggests that they have functional *VRN2* alleles. The ZCCT1 and ZCCT2 proteins from both species showed no mutations in the conserved amino acids of the CCT domains. The *Ae. speltoides* ZCCT2 protein has an H mutation at the second amino acid of the CCT domain. However, since this position is not conserved, this mutation has a small probability of disrupting the function of the *Ae. speltoides* ZCCT2 protein.

The lack of mutations in ZCCT1 and ZCCT2 in the functional *VRN2* alleles from these two diploid species is consistent with the two-ZCCT hypothesis, but it does not provide new information about the relative importance of these genes for the establishment of the vernalization requirement. The absence of mutations in the CCT domain of the *ZCCT-D1* and *ZCCT-D2* genes in diploid *Ae. tauschii* (Table I) suggests that the D genome has the potential to contribute two functional *ZCCT* copies to common wheat.

In summary, the hypothesis that both *ZCCT1* and *ZCCT2* genes can confer vernalization requirement explains well the different results on *VRN2* allelic variation described in this and previous studies.

Allelic Diversity in *VRN2* Alleles in Tetraploid Wheat

The R16C mutation in the ZCCT-A2 protein seems to be fixed in the A genome of tetraploid wheat, since it is present in all A and A^m diploid species sequences determined so far. However, the R39C mutation in the ZCCT-A1 protein is still polymorphic among the wild and cultivated *T. turgidum* subsp. *dicoccoides*. Approximately half of the accessions of these two subspecies have the R39C mutation, whereas the others do not. The R39C mutation was present in all 37 *T. turgidum* subsp. *durum* varieties analyzed in this study (Table II), suggesting that this mutation was fixed during the domestication of the modern free-threshing tetraploid wheats.

Eight of the nine *T. turgidum* subsp. *dicoccoides* accessions and all of the *T. turgidum* subsp. *dicoccon* accessions that lack the R39C mutation in ZCCT-A1 carry a R35W mutation identical to the one detected in *T. monococcum* accession DV92 (Table II). Since there is strong evidence indicating that mutations at CCT position 35 result in nonfunctional proteins (Robson et al., 2001; Yan et al., 2004b) and that the ZCCT-A2 protein from tetraploid wheat is fixed for the R16C mutation, it is very likely that most of the wild and cultivated tetraploid accessions have no functional *VRN-A2* alleles. The only possible exception was *T. turgidum* subsp. *dicoccoides* accession 10-85 from Israel (Amiad population), which showed no mutations in the CCT domain of ZCCT-A1 (Table II). We plan to cross *T. turgidum* subsp. *dicoccoides* accession 10-85 with a line homozygous for recessive *vrn-A2* and *vrn-B2* alleles to test the effect of the 10-85 *VRN-A2* allele on flowering time.

The R39C mutation in the *ZCCT-B1* gene was also polymorphic among the *T. turgidum* subsp. *dicoccon* and *T. turgidum* subsp. *dicoccoides* accessions but was fixed in all of the *T. turgidum* subsp. *durum* varieties analyzed here (Table II). On the contrary, none of the ZCCT-B2 proteins from these 37 accessions of cultivated durum wheat has mutations in the CCT domain. This result suggests that winter growth habit in cultivated tetraploid wheat is conferred mainly by the *ZCCT-B2* gene(s) and that in some *T. turgidum* subsp. *dicoccon* and *T. turgidum* subsp. *dicoccoides* accessions both the *ZCCT-B1* and *ZCCT-B2* genes can delay flowering under long days.

VRN-B2 Dosage Effect

The analysis of the progeny of BC_3F_2-521 showed that the effect of the functional *VRN-B2* locus on heading time was partially dominant (degree of dominance = 0.26), which agrees with previous results reported in barley (Szücs et al., 2007). This partial dominant effect indicates that allelic variation in the number of functional copies of *ZCCT1* and *ZCCT2* can affect heading time in tetraploid wheat. Therefore, the duplication of the functional *ZCCT-B2* gene found in most cultivated durum wheat and in some *T. turgidum* subsp. *dicoccon* accessions may have contributed to the variation in heading time in tetraploid wheat. The high sequence identity between the two copies (99.7% identity) suggests that this duplication originated recently.

The duplication of the functional *ZCCT-B2* locus provides a simple explanation for the higher transcript levels of *ZCCT2* relative to *ZCCT1* in tetraploid wheat (Fig. 7, A and B copies combined). The opposite result was observed before in *T. monococcum*, where *ZCCT-A^m1* transcripts were more abundant than those of *ZCCT-A^m2* (Yan et al., 2004b). It is interesting that in both cases the most abundant transcripts were those including the functional alleles (*ZCCT-A^m1* in *T. monococcum* accession G3116 and *ZCCT-B2* in tetraploid wheat). A possible explanation for this observation could be the progressive degradation of regulatory elements of genes that are no longer functional. Deletions or mutations in binding sites for regulatory elements in the promoters of nonfunctional alleles would have no effect on flowering time and therefore would not be affected by purifying selection.

In addition to the internal duplication of the *ZCCT-B2* gene in cultivated wheat, other deletion and duplication events affected the complete *VRN-B2* locus. The deletion of all *ZCCT* genes from the B genome found in *T. turgidum* subsp. *dicoccon* accession PI470739 was instrumental in demonstrating the dosage effect of functional *ZCCT* genes in polyploid wheat. The RFLP screening also revealed the existence of a duplication of the complete *VRN-B2* locus affecting both *ZCCT-B1* and *ZCCT-B2* genes (*T. turgidum* subsp. *dicoccoides* from Rosh Pinna). The copy number of *ZCCT-B1* and *ZCCT-B2* in these accessions is currently unknown, but the intensity of the hybridization signal suggests the presence of several copies (Fig. 2). We have initiated the crosses required to study the effect of this duplication on flowering time.

CONCLUSION

Accessions with a spring growth habit determined only by deletions or mutations in the *VRN2* locus are frequent in cultivated barley (Dubcovsky et al., 2005; Szücs et al., 2007) and diploid wheat (Yan et al., 2004b). These *VRN2* mutations are also found in combination with dominant *VRN1* alleles. These results suggest that *vrn2* mutations alone or in combination with some dominant *VRN1* alleles might confer different responses to environmental cues from those conferred by those *VRN1* mutations alone.

The discovery that durum wheat varieties have nonfunctional *vrn-A2* alleles and the development of a codominant marker tightly linked to the *vrn-B2* deletion (PI470739) will facilitate the development of spring durum wheat varieties with no functional *VRN2* loci. These nonfunctional *VRN2* alleles can then be used alone or in combination with different dominant *VRN1* alleles to develop spring durum wheat varieties with new allelic diversity in heading time.

Allelic variation for *VRN2* can be widened also in the opposite direction by adding more copies of functional *ZCCT* genes to cultivated durum wheat. This is expected to increase vernalization requirement and/or delay flowering, although its final effect will depend on other vernalization genes present in the genetic background. The *ZCCT-A1* allele with no mutations in the CCT domain (*T. turgidum* subsp. *dicoccoides* accession 10-85) can be used to replace the nonfunctional *ZCCT-A1* gene in cultivated durum wheat. In addition, the duplicated *VRN-B2* allele present in the *T. turgidum* subsp. *dicoccoides* accessions from Rosh Pinna may be deployed in cultivated durum wheat.

Allelic variation in the *ZCCT* closest homolog in rice (*Ghd7*) has shown significant contributions of this locus to both the productivity and adaptability of cultivated rice on a global scale (Xue et al., 2008). Hopefully, the ZCCT allelic diversity described here would be useful to fine-tune heading time and improve or expand the adaptability of tetraploid and hexaploid wheat varieties to different environments.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Triticum monococcum accession DV92 was the source of the nonfunctional *vrn-A^m2* allele (Yan et al., 2004b), and *Triticum turgidum* subsp. *dicoccon* accession PI470739 was the source of the recessive *vrn-B2* allele. The winter tetraploid durum variety Durelle was used as the source of the recessive *vrn-A1* and *vrn-B1* alleles. The RFLP screening included a previously described collection (Dvorak et al., 2006) including 614 wild and cultivated tetraploid accessions, 445 hexaploid accessions, and 443 diploid wheat accessions.

Seeds were imbibed for 24 h at 4°C to promote synchronized germination. Seedlings were transferred to pots and watered with nutrition solution. Unvernalized plants were grown in a greenhouse at room temperature (20°C–25°C) and long-day photoperiod (8 h of dark/16 h of light). For the vernalization experiments, plants were first grown for 3 weeks at the same

conditions described above, transferred to a cold room at 4°C and a long-day photoperiod for 4 weeks, and then transferred back to the greenhouse to score heading date. Heading date was recorded at complete spike emergence.

Methods used for sequencing BAC clone 738D05 (*VRN-B2* locus), hybridization, PCR, and quantitative reverse transcription PCR, together with the markers for *VRN-A^{m}2*, *VRN-B2*, *VRN-A1*, and *PINA* loci, are described in the Supplemental Data. Primers for all of the experiments are described in Supplemental Table S1.

Development of a Tetraploid Wheat Line Segregating for *VRN-A2* and *VRN-B2*

The following crosses and selections were performed to introduce the nonfunctional *vrn-A^{m}2* allele (R35W) from *T. monococcum* accession DV92 and the null *vrn-B2* allele from *T. turgidum* subsp. *dicoccon* PI470739 into tetraploid wheat. *T. monococcum* accession DV92 was crossed with cultivated tetraploid wheat Langdon (Fig. 3A), which carries dominant *VRN-A1* and recessive *vrn-B1* alleles (Fu et al., 2005). The hybrid from this cross was backcrossed to Langdon (Fig. 3B), and two BC_1 plants were obtained. Plant BC_1#2 was confirmed to carry the recessive *vrn-A^{m}2* allele using a cleaved amplified polymorphic sequence (CAPS) marker (see below and Fig. 3C).

Plant BC_1#2 was crossed with the tetraploid winter wheat Durelle to incorporate the recessive *vrn-A^{m}2* allele into a winter background (Fig. 3D). The BC_2F_1 plant from this cross was self-pollinated, and a population of 80 BC_2F_2 plants was generated and grown in a greenhouse without vernalization (Fig. 3E). This population showed a 3:1 (62:18) segregation between winter and spring growth habit, as expected for a population segregating only for *VRN-A1*. Winter BC_2F_2 lines (homozygous for recessive *vrn-A1* and *vrn-B1* alleles) were screened with the *VRN-A^{m}2* CAPS marker, and three lines homozygous for the recessive *vrn-A^{m}2* allele were selected (Fig. 3F).

The selected BC_2F_2 lines were crossed with *T. turgidum* subsp. *dicoccon* accession PI470739 (Fig. 3F), which is homozygous for a deletion encompassing both *ZCCT-B1* and *ZCCT-B2* genes (recessive *vrn-B2* allele). Three BC_3F_1 plants were self-pollinated, and the resulting BC_3F_2 seeds were grown in a greenhouse without vernalization to select winter BC_3F_2 plants (Fig. 3G). The winter lines (homozygous *vrn-A1* and *vrn-B1*) were then screened with the *VRN-A^{m}2* CAPS marker and with a codominant marker for *SNF-B2* (Fig. 3H), a gene tightly linked to *VRN2* (Yan et al., 2004b), to select plants heterozygous for both *VRN2* loci. Plant BC_3F_2-521 was selected, and its progeny were used for the genetic analysis presented in this study (Fig. 3I).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers FJ173816 to FJ173824 and FJ427399.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Primers and genetic markers.

Supplemental Materials and Methods S1.

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LITERATURE CITED

Akhunov ED, Akhunova AR, Dvorak J (2005) BAC libraries of *Triticum urartu*, *Aegilops speltoides* and *Ae. tauschii*, the diploid ancestors of polyploid wheat. Theor Appl Genet **111:** 1617–1622

Ben-Naim O, Eshed R, Parnis A, Teper-Bamnolker P, Shalit A, Coupland G, Samach A, Lifschitz E (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. Plant J **46:** 462–476

Bonafede M, Kong L, Tranquilli G, Ohm H, Dubcovsky J (2007) Reduction of a *Triticum monococcum* chromosome segment carrying the softness genes *Pina* and *Pinb* translocated to bread wheat. Crop Sci **47:** 821–826

Cai XN, Ballif J, Endo S, Davis E, Liang MX, Chen D, DeWald D, Kreps J, Zhu T, Wu YJ (2007) A putative CCAAT-binding transcription factor is a regulator of flowering timing in Arabidopsis. Plant Physiol **145:** 98–105

Cockram J, Chiapparino E, Taylor SA, Stamati K, Donini P, Laurie DA, O'Sullivan DM (2007) Haplotype analysis of vernalization loci in European barley germplasm reveals novel *VRN-H1* alleles and a predominant winter *VRN-H1/VRN-H2* multi-locus haplotype. Theor Appl Genet **115:** 993–1001

Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: a sequence logo generator. Genome Res **14:** 1188–1190

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, Sarhan F (2003) *TaVRT-1*, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol **132:** 1849–1860

Distelfeld A, Li C, Dubcovsky J (2009) Regulation of flowering in temperate cereals. Curr Opin Plant Biol (in press)

Dubcovsky J, Chen C, Yan L (2005) Molecular characterization of the allelic variation at the *VRN-H2* vernalization locus in barley. Mol Breed **15:** 395–407

Dubcovsky J, Dvorak J (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. Science **316:** 1862–1866

Dubcovsky J, Loukoianov A, Fu D, Valarik M, Sanchez A, Yan L (2006) Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*. Plant Mol Biol **60:** 469–480

Dvorak J, Akhunov ED, Akhunov AR, Deal KR, Luo MC (2006) Molecular characterization of a diagnostic DNA marker for domesticated tetraploid wheat provides evidence for gene flow from wild tetraploid wheat to hexaploid wheat. Mol Biol Evol **23:** 1386–1396

Dvorak J, McGuire PE, Cassidy B (1988) Apparent sources of the A genomes of wheats inferred from the polymorphism in abundance and restriction fragment length of repeated nucleotide sequences. Genome **30:** 680–689

Dvorak J, Zhang HB (1990) Variation in repeated nucleotide sequences sheds light on the phylogeny of the wheat B and G genomes. Proc Natl Acad Sci USA **87:** 9640–9644

Fu D, Szücs P, Yan L, Helguera M, Skinner J, Hayes P, Dubcovsky J (2005) Large deletions in the first intron of the *VRN-1* vernalization gene are associated with spring growth habit in barley and polyploid wheat. Mol Genet Genomics **273:** 54–65

Griffiths S, Dunford RP, Coupland G, Laurie DA (2003) The evolution of *CONSTANS*-like gene families in barley, rice, and Arabidopsis. Plant Physiol **131:** 1855–1867

Hemming MN, Peacock WJ, Dennis ES, Trevaskis B (2008) Low temperature and daylength cues are integrated to regulate *FLOWERING LOCUS T* in barley. Plant Physiol **147:** 355–366

Karsai I, Szücs P, Meszaros K, Filichkina T, Hayes PM, Skinner JS, Lang L, Bedo Z (2005) The *Vrn-H2* locus is a major determinant of flowering time in a facultative × winter growth habit barley (*Hordeum vulgare* L.) mapping population. Theor Appl Genet **110:** 1458–1466

Kihara H (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare* (Japanese). Agric Hortic (Tokyo) **19:** 13–14

Kurup S, Jones HD, Holdsworth MJ (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J **21:** 143–155

Lantican MA, Dubin HJ, Morris ML (2005) Impacts of International Wheat Breeding Research in the Developing World, 1988-2002. International Maize and Wheat Improvement Center, Mexico, D.F.

Li C, Dubcovsky J (2008) Wheat FT protein regulates *VRN1* transcription through interactions with FDL2. Plant J **55:** 543–554

Loukoianov A, Yan L, Blechl A, Sanchez A, Dubcovsky J (2005) Regulation of *VRN-1* vernalization genes in normal and transgenic polyploid wheat. Plant Physiol **138:** 2364–2373

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80:** 847–857

Robson F, Costa MMR, Hepworth SR, Vizir I, Pineiro M, Reeves PH, Putterill J, Coupland G (2001) Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. Plant J **28:** 619–631

Romier C, Cocchiarella F, Mantovani R, Moras D (2003) The NF-YB/NF-YC structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y. J Biol Chem **278:** 1336–1345

Shitsukawa N, Ikari C, Shimada S, Kitagawa S, Sakamoto K, Saito H, Ryuto H, Fukunishi N, Abe T, Takumi S, et al (2007) The einkorn wheat (*Triticum monococcum*) mutant, *maintained vegetative phase*, is caused by a deletion in the *VRN1* gene. Genes Genet Syst **82:** 167–170

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S, Kreps JA, Kay SA (2000) Cloning of the Arabidopsis clock gene *TOC1*, an autoregulatory response regulator homolog. Science **289:** 768–771

Szücs P, Skinner JS, Karsai I, Cuesta-Marcos A, Haggard KG, Corey AE, Chen THH, Hayes PM (2007) Validation of the *VRN-H2/VRN-H1* epistatic model in barley reveals that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity. Mol Genet Genomics **277:** 249–261

Takahashi R, Yasuda S (1971) Genetics of earliness and growth habit in barley. *In* RA Nilan, ed, Proceedings of the 2nd International Barley Genetics Symposium. Washington State University Press, Pullman, WA, pp 388–408

Tranquilli GE, Dubcovsky J (2000) Epistatic interactions between vernalization genes *Vrn-A^{m}1* and *Vrn-A^{m}2* in diploid wheat. J Hered **91:** 304–306

Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, Dennis ES (2003) MADS box genes control vernalization-induced flowering in cereals. Proc Natl Acad Sci USA **100:** 13099–13104

Trevaskis B, Hemming MN, Dennis ES, Peacock WJ (2007) The molecular basis of vernalization-induced flowering in cereals. Trends Plant Sci **12:** 352–357

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES (2006) *HvVRN2* responds to daylength, whereas *HvVRN1* is regulated by vernalization and developmental status. Plant Physiol **140:** 1397–1405

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. Science **310:** 1031–1034

vonZitzewitz J, Szücs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen THH, Hayes PM, Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. Plant Mol Biol **59:** 449–467

Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, Coupland G (2006) CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. Plant Cell **18:** 2971–2984

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309:** 1056–1059

Xue WY, Xing YZ, Weng XY, Zhao Y, Tang WJ, Wang L, Zhou HJ, Yu SB, Xu CG, Li XH, et al (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. Nat Genet **40:** 761–767

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. Proc Natl Acad Sci USA **103:** 19581–19586

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004a) Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor Appl Genet **109:** 1677–1686

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004b) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science **303:** 1640–1644

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of wheat vernalization gene *VRN1*. Proc Natl Acad Sci USA **100:** 6263–6268