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Genetic and Molecular Characterization of the *VRN2* loci in Tetraploid Wheat

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ABSTRACT

Winter wheat 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. However, natural allelic variation in these genes has not been described so far in polyploid wheat. A tetraploid wheat population segregating for both *VRN-A2* and *VRN-B2* loci facilitated the characterization of different alleles. Comparisons between functional and non-functional 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 non-functional *vrn2* alleles have mutations at arginine amino acids at positions 16, 35 or 39 of the CCT domain. These positions are conserved between CCT and HAP2 domains 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.

INTRODUCTION

Wheat 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) are *VRN1*, *VRN2* and *VRN3*. *VRN1* is a homologue of the Arabidopsis meristem identity gene *AP1*, 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* L. (2n=14, A^m genome similar to 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; Yan et al., 2004a; Fu et al., 2005; vonZitzewitz et al., 2005).

VRN3 is a homologue of Arabidopsis photoperiod gene *FT*, 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 et al. 2008). Before vernalization, *VRN3* is down-regulated by *VRN2* preventing winter wheats to flower 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 and Distelfeld et al., 2009). The focus of this paper is the natural variation in *VRN2*.

In diploid wheat, the *VRN2* locus includes two tandemly duplicated genes designated as *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 function 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), which is not observed in control plants kept at non-vernalizing 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 (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 (RNAi) of *ZCCT1* in hexaploid winter wheat variety Jagger (*Triticum aestivum* L. 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 *T. turgidum* L. (2n=28, genomes AABB), the donor of the A and B genomes to hexaploid wheat. Tetraploid wheats are divided into three subspecies: *T. turgidum* ssp. *dicoccoides* (wild accessions with disarticulating spike), *T. turgidum* ssp. *dicoccon* (partially domesticated, with non-disarticulating spikes and non free-threshing grains) and *T. turgidum* ssp. *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*, *ZCCT1*, *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, AY485644) and the A genome of tetraploid wheat *T. turgidum* ssp. *durum* variety Langdon (Dubcovsky and Dvorak, 2007, EF540321). A low-coverage sequencing of BAC 738D05 including the *VRN-B2* locus from *T. turgidum* ssp. *durum* revealed three *ZCCT* genes, designated as *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 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 accession of diploid *Triticum* and *Aegilops* species with genomes similar to the A, B and D genomes of hexaploid wheat. These species included *T. urartu* Tumanian ex Gandilyan, the donor of the A genome (Dvorak et al., 1988); *Ae. speltoides* Tausch (S genome), the closest extant diploid species to the B genome of tetraploid and hexaploid wheat (Dvorak and Zhang, 1990); and *Ae. 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. *Aegilops 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 one 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.

RFLP germplasm screen

The hybridization of Southern blots including *Dra*I digested DNAs from wild and cultivated tetraploid and hexaploid *Triticum* accessions (see Material 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 (≈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 81-82% 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 and 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* ssp. *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 in 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* ssp. *dicoccon* (PI470739) collected in the mountains of Kars, Turkey (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 10-11). 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 (Table SI, supporting online materials). These primers failed to amplify any *ZCCT* fragment from PI470739.

Molecular characterization of the 5A-5A^{m} translocation in BC3F2-521

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 non-functional *vrn-A^{m}2* from *T. monococcum* accession DV92. The development of this plant is described in Materials and Methods and in Fig. 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 for 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 arm 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 indicated 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 Fig. 3J.

Effect of the allelic differences in *VRN-A2* and *VRN-B2* on flowering time

The non-vernalized progeny of line BC_3F_2-521 segregated into two non-overlapping groups for flowering time. The first group included 13 early-flowering plants that headed in less than 60 days (average 53.0 ± 0.4 days) and were classified as spring, whereas the second group included 41 late-flowering plants that took more than 90 days for heading (average 139.1 ± 4.6 days) 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 to 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, χ^2=29.3, P<0.0001), but not from a 1:3 ratio (1 dominant gene, χ^2=0.025, P=0.88). The large differences in heading time observed among the non-vernalized 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 (supporting online materials) 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 (Table SI, supporting online materials). 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 days after sowing, whereas those heterozygous for *VRN-B2* flowered significantly ($P<0.001$) earlier (120 ± 4.3 days after sowing). Heading time of the heterozygous plants was only 14 days later than the mid-point between the two homozygous classes (106 d). Using these numbers, the degree of dominance was calculated to be 0.26 (14d/53d, 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) as the vernalized plants homozygous for the *vrn-B2* deletion (74.1 ± 1.2, $P=0.26$). 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 non-functional *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 a 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 non-functional *VRN-A2* locus from BC_3F_2-521 has a 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 domains (Fig. 6).

The R39C mutation was detected in all 37 cultivated *T. turgidum* ssp. *durum* accessions analyzed in this study, but was polymorphic in cultivated *T. turgidum* ssp. *dicoccon* (present in 11 out of 22 accessions) and wild *T. turgidum ssp. dicoccoides* (present in 10 out of 19 accessions, Table II). One accession from Asia Minor (PI355454) showed an additional R35Q mutation. The eleven accessions of *T. turgidum* ssp. *dicoccon* that lack the R39C mutation all have a R35W mutation. The R35W mutation was also found in 8 out of the 9 accessions of *T. turgidum ssp. dicoccoides* that do not have the R39C mutation. *T. turgidum ssp. 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^m1 protein from *T. monococcum*. These 7 amino acids are located immediately downstream of the putative zinc finger domain from amino acids 49 to 55 (numbers are relative to the initial methionine in ZCCT-A^m1). A screening using primers VRN2/22F + R (Table SI, supporting online materials) showed that the same deletion was present in all 78 tetraploid wheats tested in this study (Table II) and in 103 out of 107 *T. urartu* accessions. The *ZCCT-A1* genes from 15 *T. urartu* accessions were fully sequenced and showed that none of them have mutations in the CCT domain.

ZCCT-A2: The predicted ZCCT-A2 protein corresponding to the non-functional *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 genes (except for CO-like Group II) and HAP2 domains (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 out of the four *T. monococcum* accessions (including DV92) have an R39C mutation in addition to the R16C mutation in ZCCT-A^m2.

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 non-functional *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 ssp. dicoccoides* (present in 4 out of 19 accessions) and cultivated *T. turgidum* ssp. *dicoccon* (present in 4 out 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 (Table SI, supporting online materials) failed to detect the duplication in *T. turgidum* ssp. *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 the *T. turgidum* ssp. *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* ssp. *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* ssp. *durum* varieties (36 out 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 three, four and five-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 RTPCR primers (supporting online materials) were designed to differentiate *ZCCT1* from *ZCCT2* but not A from B genome copies of the same gene, the transcript levels presented in Fig. 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 non-functional *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 has been recently shown that the CCT domains from Arabidopsis CO and COL15 can interact with several *At*HAP3 and *At*HAP5 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 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 have 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) providing additional support to this hypothesis.

Interestingly, the three CCT mutations identified here in ZCCT proteins coded by genes located in non-functional *VRN2* loci are located at positions 16, 35 and 39, which are conserved both between and within the CCT and HAP2 domains (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 arginine 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 and therefore, with high probability of altering protein structure and function.

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 hereafter to this model 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.

1.- 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 ZCCT2 wheat proteins (and 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.

2.- Non-functional *vrn2* alleles: The two-ZCCT hypothesis predicts that all recessive *vrn2* alleles would have non-functional 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 7 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 non-functional 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 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. (2004) concluded that *ZCCT-A^m1* was *VRN-2* and that *ZCCT-A^m2* was not important for the determination of the winter growth habit (Yan et al., 2004b). At that point, it was not clear that the *ZCCT-A^m2* gene in *T. monococcum* was fixed for a non-functional allele, simplifying the detection of the segregation for the R35W mutation in *ZCCT-A^m1*.

Although the published conclusion is valid for *T. monococcum*, the current results indicate that it cannot be generalized to all *Triticeae* species.

3.- 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 accession (AY485976, 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 non-functional *VRN-A2* allele (Table I). The low BLOSUM62 score (-3) and the fact that this mutation alters a conserved position across HAP2 and CCT domains (Fig. 6) suggest that this ZCCT-B1 protein is non-functional. 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.

4.- 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 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 the A and A^m diploid species sequences so far. However, the R39C mutation in the ZCCT-A1 protein is still polymorphic among the wild and cultivated *T. turgidum* ssp. *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* ssp. *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* ssp. *dicoccoides* accessions and all the *T. turgidum* ssp. *dicoccon* 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 non-functional proteins (Robson et al., 2001; Yan et al., 2004b) and 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* ssp. *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 ssp. *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* ssp. *dicoccon* and *T. turgidum* ssp. *dicoccoides* accessions but was fixed in all the *T. turgidum* ssp. *durum* varieties analyzed here (Table II). On the contrary, none of the ZCCT-B2 proteins from these 37 accessions of cultivated durum 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* ssp. *dicoccon* and *T. turgidum* ssp. *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 and in some *T. turgidum* ssp. *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 was 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^{m}1* transcripts were more abundant than those of *ZCCT-A^{m}2* (Yan et al., 2004b). It is interesting to point out here, that in both cases the most abundant transcripts were those including the functional alleles (*ZCCT-A^{m}1* 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 non-

functional 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* ssp. *dicoccon* accession PI470739 was instrumental to demonstrate 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* ssp. *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.

CONCLUSIONS

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 non-functional *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 non-functional *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* ssp. *dicoccoides* accession 10-85) can be used to replace the non-functional *ZCCT-A1* gene in cultivated durum wheat. In addition, the duplicated *VRN-B2* allele present in the *T. turgidum* ssp. *dicoccoides* accessions from Rosh Pinna population may be deployed in cultivated durum wheat.

Allelic variation in the ZCCT closest homologue in rice (*Ghd7*) has shown significant contributions of this locus to both productivity and adaptability of cultivated rice on a global scale (Xue et al., 2008). Hopefully, a larger diversity of functional *ZCCT* copies may 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

T. monococcum accession DV92 was the source of the non-functional *vrn-A^{m}2* allele (Yan et al., 2004b) and *T. turgidum* spp. d*icoccon* 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 24h 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 (20-25°C) and long-day photoperiod (8 h dark / 16 h light). For the vernalization experiments, plant were first grown for three weeks at the same conditions described above, transferred to a cold room at 4°C and 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.

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Methods used for sequencing BAC clone 738D05 (*VRN-B2* locus), hybridization, polymerase chain reaction (PCR) and quantitative reverse transcription PCR (Q-RTPCR); together with the markers for *VRN-A^{m}2*, *VRN-B2*, *VRN-A1* and *PINA* loci are described in the supporting online materials. Primers for all the experiments are described in Table SI, also available on the supporting online materials.

Development of a tetraploid wheat line segregating for *VRN-A2* and *VRN-B2*

The following crosses and selections were performed to introduce the non-functional *vrn-A^{m}2* allele (R35W) from *T. monococcum* accession DV92 and the null *vrn-B2* allele from *T. turgidum* ssp. *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 CAPS marker (Cleaved Amplified Polymorphic Sequence, see section 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* ssp. *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 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 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).

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FIGURE LEGENDS

Fig. 1. **Neighbor Joining phylogenetic analyses of wheat ZCCT proteins**. Bootstrap values based on 1000 replications are indicated above their respective nodes. Only values above 50 are presented.

Fig. 2. RFLP screening of polyploid *Triticum* accessions for variation in *ZCCT* genes. **Lanes 1-7**: *T. turgidum* ssp. *durum* accessions PI60727, PI60730, PI60731, PI60732, PI60733, PI7016, and PI10388. **Lane 8**: *T. turgidum* ssp. *dicoccoides* accession PI428107 (Rosh Pinna, Israel). Note the increased hybridization signal in fragments corresponding to *ZCCT-B1* and *ZCCT-B2* as compared to the non duplicated *ZCCT-A2* 1,420 bp band in the same accession. **Lanes 9-13**: *T. turgidum* ssp. *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*.

Fig. 3. Generation of a tetraploid line segregating for *VRN-A*2 and *VRN-B2*. A) Introgression of the non-functional *vrn-A^m*2 allele from DV92 into tetraploid wheat. Black bars represent *T. monococcum* chromosomes and gray bars *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 selected winter BC_2F_2 plant carrying the *vrn-A^m*2 allele to *T. turgidum* ssp. *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, (W)= Winter growth habit.

Fig. 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)** Un-vernalized plants (1: 3 segregation spring to winter growth habit). **B)** Vernalized plants (no segregation for flowering time).

Fig. 5. Effect of *VRN-A2* and *VRN-B2* alleles on heading time in non-vernalized 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 ssp. 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 indicated alleles.

Fig. 6. Alignment of the CCT domains from different plants 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 non-mutant *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. *Tm= T. monococcum. Ae. spelt.= Aegilops speltoides*. **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.

Fig. 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* ssp. *dicoccon* accession PI470739 and *Vrn-B2* from varieties Langdon or Durelle). Eight plants were sampled from 3, 4 and 5 weeks old plants. Gray bars represent *ZCCT2* and black bars represent *ZCCT1* transcripts. Amplification primers are conserved for the A and B homoeologous copies of each gene. ** and *** indicate significant differences (t-test) at $P \leq 0.01$ or 0.001, respectively.

Table I. Summary of polymorphisms between ZCCT1 and ZCCT2 proteins from different species and genomes. Numbers below the protein name indicate the position of the amino acid in the CCT domain. del= 7 amino acid deletion.

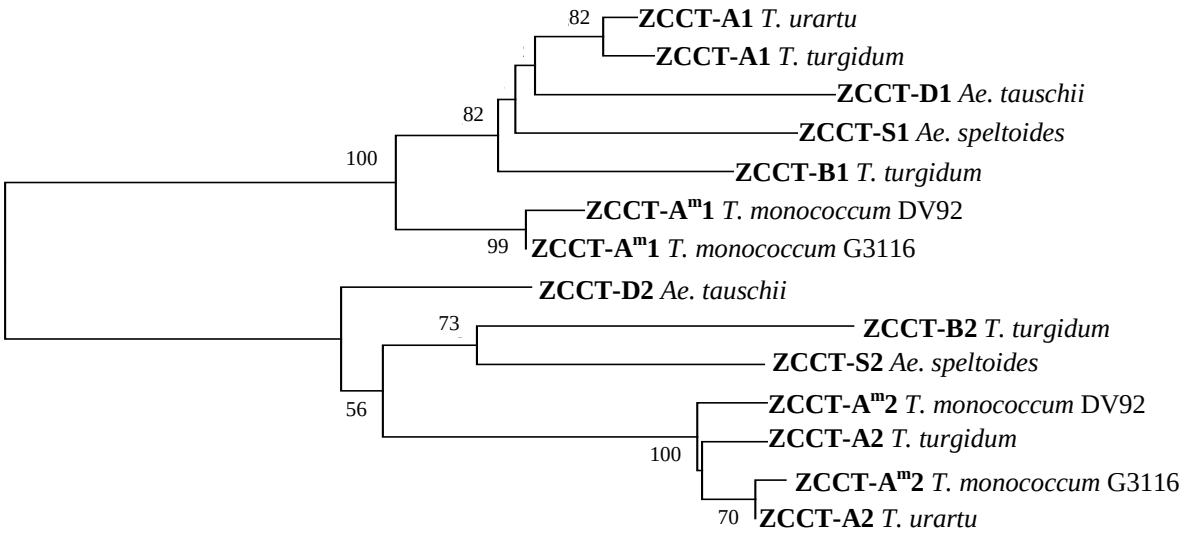
Genotype	VRN2 function	ZCCT1				ZCCT2		
		del	**16**	**35**	**39**	**16**	**35**	**39**
T. urartu	Functional [1]	yes	R	R	R	**C**	R	R
Ae. speltoides	Functional [1]	No	R	R	R	R	R	R
Ae. tauschii	Functional [1]	No	R	R	R	R	R	R
T. monococcum 3116	Functional	No	R	R	R	**C**	R	R
T. monococcum DV92	Non-functional	No	R	**W**	R	**C**	R	**C**
T. turgidum 521 A genome	Non-functional	yes	R	R	**C**	**C**	R	R
T. turgidum 521 B genome	Functional	No	R	R	**C**	R	R	R

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

Table II. Summary of different *ZCCT-A1* and *ZCCT-B1* alleles in a collection of wild *T. turgidum* ssp. *dicoccoides*, partially domesticated *T. turgidum* ssp. *dicoccon* and modern cultivated *T. turgidum* ssp. *durum*. PI accessions are from the National Small Grain Collection (USA) and other numbers are population numbers from the University of Haifa. The number below the gene indicates its position within the CCT domain. All ZCCT-A1 and ZCCT-B1 proteins included in this table have an R at position 16.

Germplasm	Country	Species	ZCCT-A			ZCCT-B		
			A1		A2	B1		B2
			35	39	16[1]	35	39	No
10-85	Israel	*T. turgidum* ssp. *dicoccoides*	R	R	**C**	R	R	1[2]
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		**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	*T. turgidum* ssp. *dicoccon*	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, USA	*T. turgidum* ssp. *durum*	R	**C**	**C**	R	**C**	**2**
Messapia	Italy		R	**C**	**C**	R	**C**	1

[1] Since the R16C mutation is fixed in *ZCCT-A2* and *ZCCT-A^{m}2* we checked only one accession from each group for this mutation. [2] The "1" indicates that only one of the two polymorphic sites was detected but does not completely rule out the existence of the duplication.



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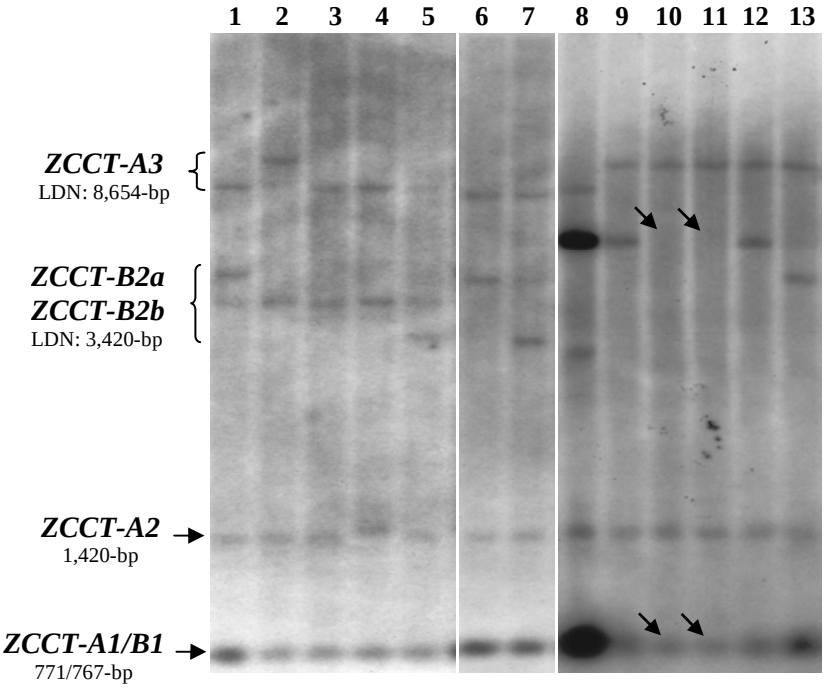

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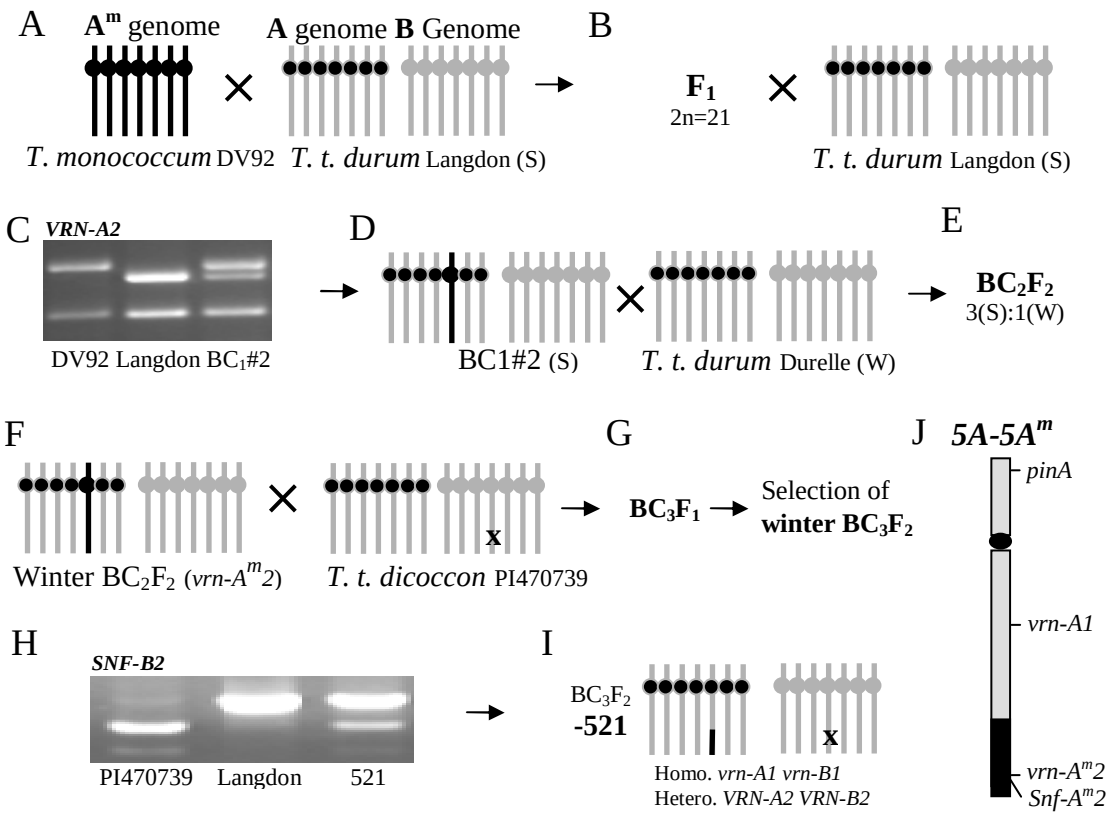

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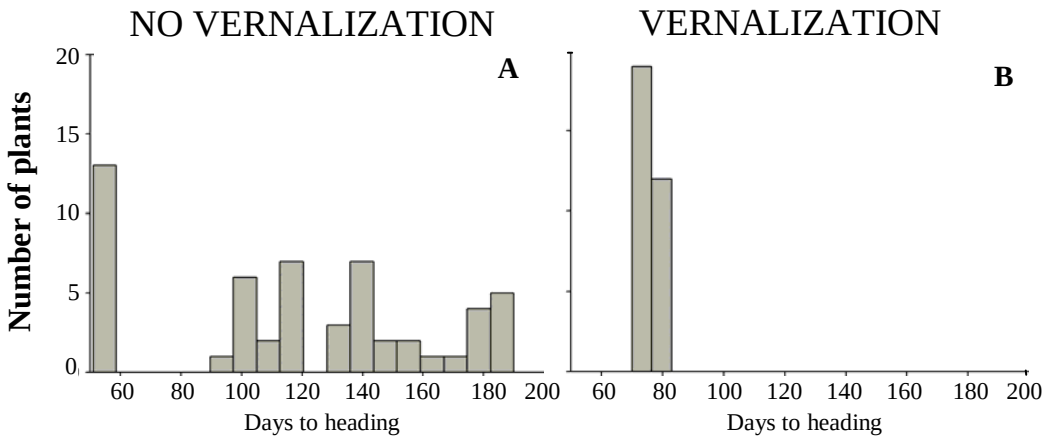

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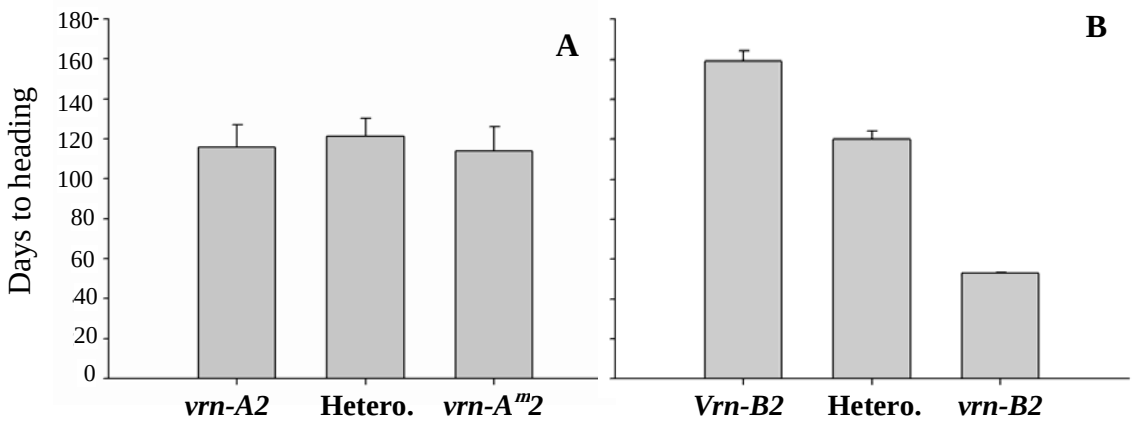

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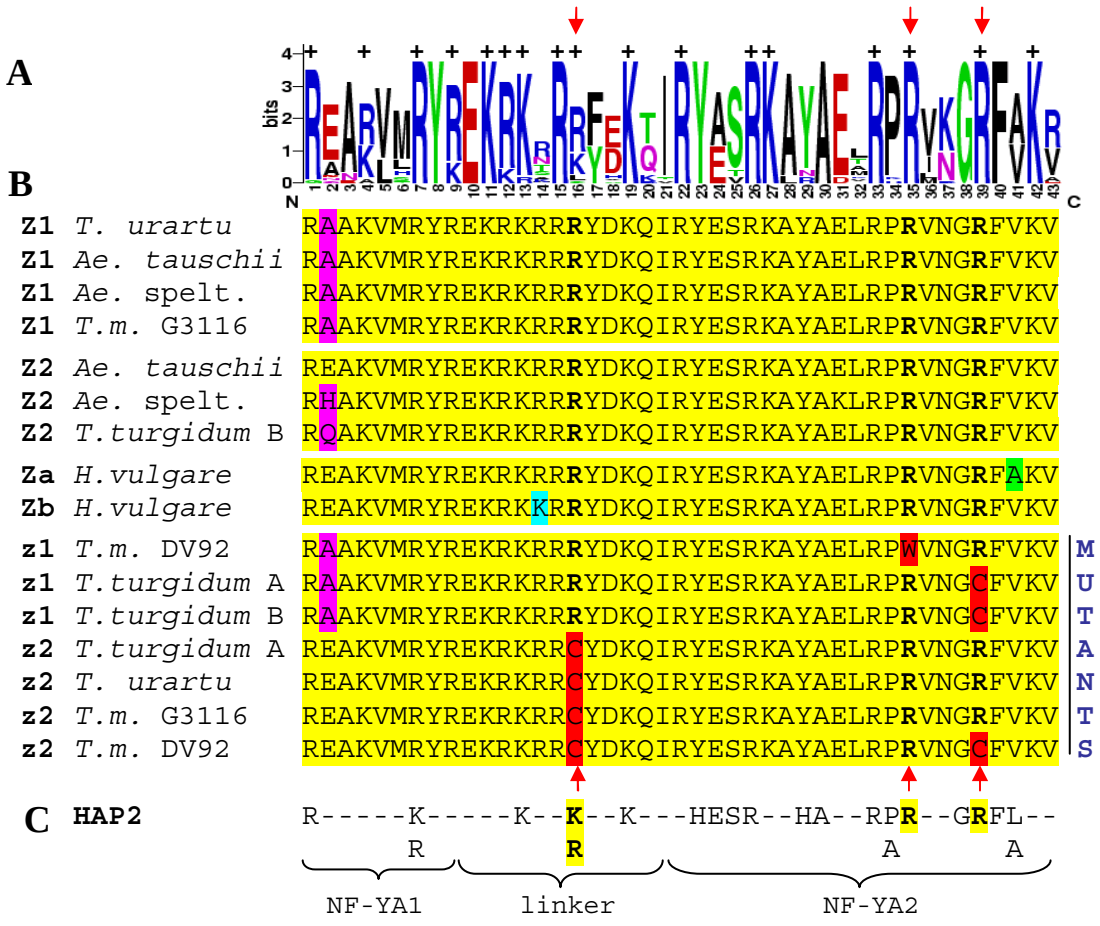

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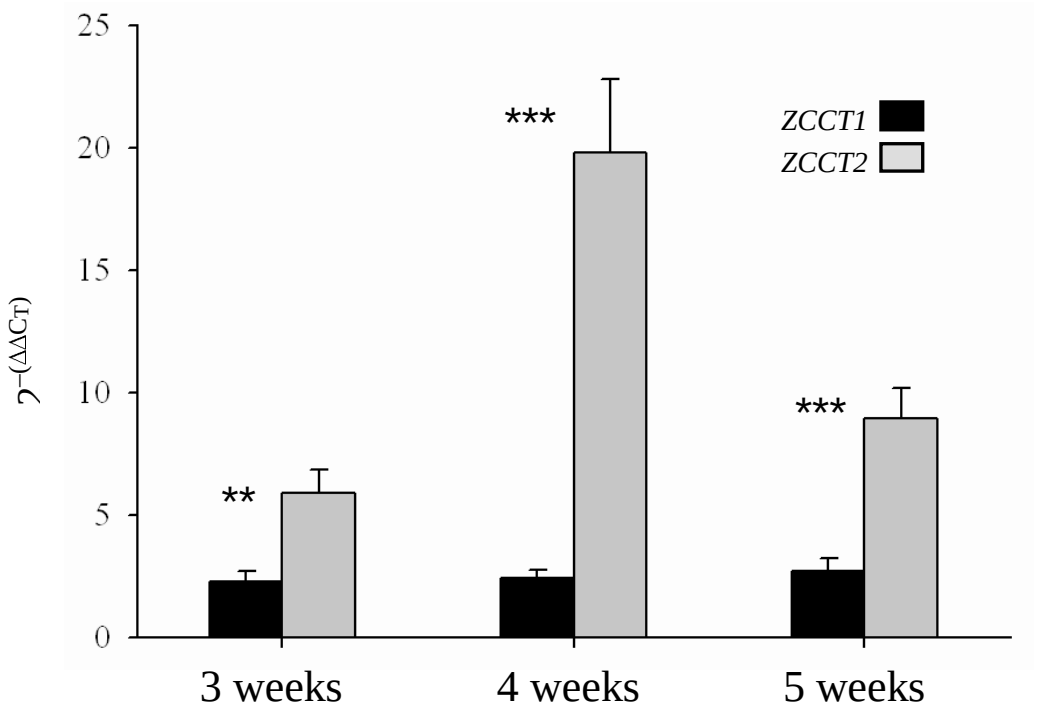

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