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The wheat *VRN2* gene is a flowering repressor downregulated by vernalization

Liuling Yan[1], **Artem Loukoianov**[1], **Ann Blechl**[3], **Gabriela Tranquilli**[1,2], **Wusirika Ramakrishna**[4], **Phillip SanMiguel**[5], **Jeffrey L. Bennetzen**[6], **Viviana Echenique**[1,7], and **Jorge Dubcovsky**[1]

[1]Dept. of Agronomy & Range Science, University of California, Davis, CA 95616, USA.

[3]USDA-ARS, Albany, CA 94710, USA.

[4]Dept of Biological Sciences, Michigan Tech University, Houghton, MI 49931, USA.

[5]Purdue University Genomics Core, Purdue University, West Lafayette, IN 47907, USA.

[6]Dept. of Genetics, University of Georgia, Athens, GA 30602, USA.

Abstract

Plants with a winter growth habit flower earlier when exposed for several weeks to cold temperatures, a process called vernalization. We report here the positional cloning of the wheat vernalization gene *VRN2*, a dominant repressor of flowering that is downregulated by vernalization. Loss of function of *VRN2*, whether by natural mutations or deletions, resulted in spring lines, which do not require vernalization to flower. Reduction of the RNA level of *VRN2* by RNA interference accelerated flowering time of transgenic winter wheat plants more than a month.

Common wheat (*Triticum aestivum* L.) is one of the primary grains consumed by humans and is grown in very different environments. This wide adaptability has been favored by the existence of wheat varieties with different growth habits. Winter wheats require a long exposure to low temperatures to flower (vernalization) and are sown in the fall, whereas spring wheats do not have a vernalization requirement and can be planted in spring or fall. The genes from the vernalization pathway prevent flower development during the winter, providing protection for the temperature-sensitive floral organs against the cold.

VRN1 and *VRN2* are the central genes in the vernalization pathway in wheat, barley, and other temperate cereals. These two genes have strong epistatic interactions and are likely part of the same regulatory pathway (1, 2). In both diploid wheat (*Triticum monococcum* L.)

Corresponding author: Jorge Dubcovsky. Fax: (530) 752 4361, jdubcovsky@ucdavis.edu.
[2]Current address, Inst. Rec. Biológicos, INTA, (1712) Castelar, Buenos Aires, Argentina.
[7]Current address, Dept. de Agronomía, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina.
The first and last authors contributed equally to this work.

Supporting Online Material
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Figs. S1 to S17, Table S1

and barley, *VRN1* is dominant for spring growth habit whereas *VRN2* is dominant for winter growth habit. Similar epistatic interactions and map locations indicate that wheat and barley vernalization genes are orthologous (3, 4).

The *VRN1* gene from wheat is similar to the Arabidopsis meristem identity gene *APETALA1* (*AP1*) (5), which initiates the transition from the vegetative to reproductive apex. Natural allelic variation at the *AP1* locus is associated with differences in vernalization requirement in wheat but not in *Arabidopsis* (5). In Arabidopsis, natural variation for vernalization requirement has arisen through the generation of non-functional or weak *FRI* and *FLC* alleles (6).

The Arabidopsis *FLC* gene is a repressor of flowering that integrates the signals from the autonomous flowering pathway with those from extended cold treatment. *FRI* elevates expression of *FLC* to levels that inhibit flowering, whereas vernalization produces a permanent downregulation of *FLC* and induces flowering (7–9).

Induced mutations have been used to identify additional vernalization genes in Arabidopsis. *VIN3* is induced by vernalization and is involved in the transient repression of *FLC* by histone deacetylation (10). Two additional Arabidopsis genes, designated *VRN1* and *VRN2*, are required to keep *FLC* in its repressed state, but not for its initial repression by cold (11, 12). Arabidopsis *VRN1* and *VRN2* are different from the wheat genes with the same names. The *VRN1* and *VRN2* names are standard designations for the wheat vernalization loci, and we will use them hereafter to refer to the cereal genes.

Positional cloning of the wheat vernalization gene *VRN2*

The *VRN2* gene was initially mapped on chromosome 5A in a small F_2 population from the cross between spring (DV92) and winter (G3116) accessions of diploid wheat *T. monococcum* (4). We used the same parental lines to develop a high-density map based on 5,698 gametes (Supporting Online Material, SOM-1). Screening of this population with *VRN2* flanking markers *NUCELLIN* and *UCW22* resulted in the identification of 18 recombination events within this region (Fig. 1 and SOM-1). Progeny tests for vernalization requirement were performed for these 18 plants. We generated additional markers from the BAC clones included in the physical map (Fig. 1) and defined the location of the two crossovers flanking *VRN2* (SOM, Figs. S2, S3 and S4). The *VRN2* gene was mapped into a 0.04-cM interval flanked by RFLP marker *UCW22* and PCR marker *UCW2.1* (Fig. 1).

Markers *UCW22* and *UCW2.1* were used to screen the BAC library of *T. monococcum* accession DV92 (13). A physical map of the *VRN2* region was constructed by chromosome walking (Fig. 1). Overlapping BACs 258C22, 301G15, 405L8, and 455C17 were sequenced (438,828-bp), annotated and deposited in GenBank (AY485644). We also sequenced orthologous BAC 615K1 from barley variety Morex (AY485643) and BAC 49F5 from rice variety Nipponbare (AF485811).

Eight genes and one pseudogene were detected within the sequenced region indicating a gene density of one gene per 55-kb and a ratio between genetic and physical distances of approximately 1.7-Mb/cM. Five of these genes were found in the same order and orientation

in the barley BAC and three in the rice BAC, confirming the colinearity of this region among cereals (Fig. 1). The closest common genes flanking the *VRN2* gene, *PDS* and *SNF2P*, were 7-kb apart in rice, 26-kb apart in barley, and 328-kb apart in *T. monococcum* (Fig.1).

The sequences from markers *UCW22* and *UCW2.1* flanking the *VRN2* gene in the genetic map (Fig. 1) were used to delimit a 315-kb candidate region within AY485644. Approximately 75% of this sequence was annotated as repetitive elements. Within the non-repetitive region only three genes were completely linked to *VRN2*. The first gene, designated *AY485644.3*, encoded a predicted 254-amino acid protein that was 87% and 96% similar to the putative orthologous proteins in the colinear BACs from rice and barley respectively (Fig. 1). The function of gene *AY485644.3* is currently unknown.

The other two genes completely linked to *VRN2* encoded proteins that were 76% identical, suggesting a duplication event that occurred approximately 14 ± 3 million years ago (SOM-2). These two proteins had similarities with *CO* and *CO*-like proteins of Arabidopsis (E= $2e^{-11}$) and rice (AP005307, *OsI*E= $3e^{-16}$; AAL79780, *OsH*E= $2e^{-16}$). This similarity was restricted to the 43-amino acids of the CCT (**C**O, **C**O-like, and **T**OC1) domain present in all these proteins (SOM-3, Fig. S10). This domain determines the nuclear localization of *CO*, the key gene in Arabidopsis photoperiod pathway (14, 15) and may have a similar function in these two genes. We named these two genes *ZCCT1* and *ZCCT2* based on the presence of a putative zinc finger in the first exon and the CCT domain in the second exon.

Evolutionary relationships between the *ZCCT* and *CO*-like genes

We isolated similar *ZCCT* genes from the A genome of tetraploid wheat (AY485979, AY485980) and from winter barley variety Diarokkaku (AY485977, AY485978), and compared their CCT domains with those from other *CO*-like genes (SOM-3, Fig. S10). We performed a Neighbor Joining cluster analysis using the CCT motifs from the *ZCCT* proteins and from members of each of the four major classes (I to IV) of *CO*-like proteins (16) (SOM-3, Fig. S11). CCT motifs from Group III (*AtCOL9* and *OsN*) were used as an out-group. The *ZCCT* proteins formed a separate group that did not include any rice or Arabidopsis protein. This group was related to Group IV proteins (*HvCO9, OsI, OsH*), which included proteins only from grass species. Proteins from both Arabidopsis and grass species were present in the separate Groups I (*AtCO, OsHD1*) and II (*AtCOL6, OsJ*), including proteins involved in the regulation of flowering by photoperiod (SOM-3, Fig. S11). Analysis of the putative zinc fingers confirmed the classification based on the CCT domains (SOM-3, Fig. S12). *CO*-like proteins from Groups I and II have one or two B-box zinc fingers whereas the *ZCCT* proteins showed one C_2H_2 zinc finger. Group IV zinc fingers were more similar, although not identical, to those from the *ZCCT* proteins (SOM-3, Fig. S12).

These results suggest that the ancestor of the *ZCCT* and Group IV proteins originated in the grasses and that the *ZCCT* proteins diverged substantially in the temperate cereals species adapted to the cold regions. The divergence of the *ZCCT* proteins might have been favored by a duplication of the ancestral Group IV protein in the temperate cereals. This is suggested

by the absence of *ZCCT* orthologues in rice in the colinear region between *AY485644.3* and *SNF2P* (Fig. 1).

Variation of the RNA levels of the candidate genes during vernalization

The RNA levels of the three genes completely linked to *VRN2* were investigated during vernalization. RNA amounts of the *AY485644.3* gene were not affected by vernalization (Fig. 2A), and were similar in spring and winter genotypes. Numerous Triticeae ESTs from different cDNA libraries showed similarity to *AY485644.3* ($E < e^{-100}$), suggesting relatively high mRNA levels in different tissues.

In contrast, the absence of ESTs corresponding to the *ZCCT* genes in the extensive wheat and barley collections suggested low transcript levels. *ZCCT1* and *ZCCT2* transcripts were detected by RT-PCR in the leaves before but not after vernalization (Fig. 2A). The mRNA levels of *ZCCT1* and *ZCCT2* in diploid wheat were quantified using TaqMan systems (SOM-4) and *ACTIN* and *UBIQUITIN* as endogenous controls (5). A progressive decrease of *ZCCT1* (Fig. 2B) and *ZCCT2* transcripts (SOM-4, Fig. S17) was observed in the leaves during vernalization. Control winter plants kept at room temperature maintained stable *ZCCT* RNA levels. *ZCCT* transcription was not restored after the plants were removed from the cold room (4°C) after six weeks of vernalization (Fig. 2B, 2w out). Downregulation of *ZCCT1* after vernalization was also confirmed in common winter wheat variety Jagger (data not shown).

The downregulation of the *ZCCT* genes during vernalization was concomitant with an increase of wheat *VRN1* (=*AP1*) transcription (Fig. 2B). These opposite transcription profiles are consistent with the epistatic interactions between *VRN1* and *VRN2* (2, 5).

ZCCT1 transcripts were present in the apices from the unvernalized winter plants but were not detected after vernalization (Fig. 2C). *VRN1* transcripts in the apices showed the same pattern as in the leaves, being induced after vernalization (Fig. 2C). Using the same RNA samples, we did not detect transcripts of *ZCCT2*. These results suggested that either *ZCCT2* was not expressed in the apices or its transcription level was below our detection threshold. Since apices are the critical points for the transition between the vegetative and reproductive phases, these observations suggested that *ZCCT1* was a better candidate for *VRN2* than was *ZCCT2*.

Allelic variation of candidate genes among cultivated *T. monococcum* accessions

ZCCT1 transcription was downregulated during vernalization in both winter G3116 and spring DV92 plants, suggesting that the differences in growth habit were not caused by differences in the transcriptional regulation of *ZCCT1*. To test this hypothesis we compared the sequences of the promoter and coding regions from the three *VRN2* candidate genes between spring and winter accessions of cultivated *T. monococcum* (SOM, Table S1).

We observed no differences in the *AY485644.3* protein between *vrn2*-spring accession DV92 and *Vrn2*-winter accessions PI355532 and PI277133 (AY485962, AY485961).

Similarly, no differences were found in the predicted *ZCCT2* proteins between *vrn2*-spring accession DV92 and *Vrn2*-winter accessions PI272561 and PI277133 (AY485976, AY485975). In addition, no differences were found between DV92 and winter accession PI272561 in the first 1-kb of the promoter or in the 763-bp of the 3' end region of the *ZCCT2* gene (SOM-5.2). These results suggested that the differences in vernalization requirement were not caused by differences in the coding sequences of these two genes or in the regulatory sequences of *ZCCT2*.

No differences were found in the promoter region of *ZCCT1* between DV92 and winter accession PI272561 (SOM-5.3). However, comparison of the *ZCCT1* coding region between DV92 and 16 *T. monococcum* accessions with winter growth habit (SOM, Table S1) provided good evidence that *ZCCT1* was the *VRN2* gene. The spring accession DV92 carried a point mutation at position 35 of the CCT domain that replaced an arginine (R) amino acid by a tryptophan (W). This arginine is conserved in all of the *ZCCT* proteins (SOM-3, Fig. S10) and in all of the *CO-like* proteins from Arabidopsis, rice and barley (16). A point mutation at the same position in the CCT domain of *CO* in Arabidopsis EMS mutant co-7 did not affect the nuclear localization of the *CO* protein but produced a severe effect on flowering time (15). Kurup et al. (17) suggested that the CCT domain might be involved in protein-protein interactions, and that a mutation within this domain can disrupt these interactions. The conservation of the 35-R amino acid in all the CCT domains and the strong effect of its mutation on flowering time in Arabidopsis suggest that this amino acid is essential for the correct function of the CCT domain, and that the point mutation observed in DV92-*ZCCT1* is the likely cause of its spring growth habit.

The R/W mutation in DV92 created an *Nco*I restriction site (SOM, Fig. S3) that was absent in the wild allele. This polymorphism was used to screen a germplasm collection of 65 accessions of cultivated *T. monococcum* from different parts of the world (SOM, Table S1). The R/W mutation was absent in all 16 accessions with a winter growth habit, but present in 22 of the 49 spring accessions (SOM, Table S1). Screening of the remaining 27 spring accessions by hybridization with *ZCCT1* showed that 17 accessions had a complete deletion of *ZCCT1* and *ZCCT2*. Seven of the ten remaining spring accessions showed deletions in the *VRN1* promoter that explained their spring growth habit (5). The spring growth habit of the last three accessions remains unexplained. In summary, the mutations or deletions at the *ZCCT1* gene and the *VRN1* promoter described so far were sufficient to explain the spring growth habit of 46 out of the 49 cultivated *T. monococcum* accessions analyzed in this study (SOM, Table S1).

Allelic variation in barley

The absence of *ZCCT* genes in the orthologous BAC from spring barley variety Morex (Fig. 1) suggested that this variety carries a recessive *vrn2* allele. Hybridization of *Xba*I digested barley genomic DNA with a wheat *ZCCT1* probe showed no hybridization in Morex, but three RFLP fragments in winter *H. spontaneum*. The analysis of 102 F_2 plants from a cross between the spring variety Morex and the winter accession of *H. spontaneum* showed that all the F_2 plants homozygous for the deletion had a spring growth habit. In addition, the three RFLP fragments were completely linked to *VRN2* flanking gene *SNF2P*,

demonstrating that Morex has a recessive *vrn2* allele completely linked to the *ZCCT* deletion.

To study the distribution of the deletion of the *ZCCT* genes in barley and its association with the *vrn2* allele, we screened a collection of 85 barley varieties from different parts of the world that were previously characterized genetically for their vernalization alleles (18). Hybridization of Southern blots of DNAs from these varieties with the wheat *ZCCT1* probe showed the presence of the *ZCCT* genes in the 23 winter barley varieties and complete deletion of all *ZCCT* genes in 61 *vrn2*-spring barley varieties (19). Spring barley variety Fan (*vrn2*) showed only one *ZCCT* gene, indicating a different deletion (19).

Validation of *ZCCT1* as *VRN2* by RNAi transgenic wheats

Transformation experiments were performed in winter hexaploid wheat variety Jagger because it is currently not possible to transform efficiently diploid *T. monococcum*. Transformation was performed by bombardment with an RNA interference (RNAi) construct including a 347-bp segment from the *T. monococcum ZCCT1* gene (SOM-6). We identified three independent transgenic plants by PCR (SOM-6), but only one of them showed the expected acceleration of flowering relative to the negative controls and was analyzed further.

We self-pollinated the early-flowering transgenic T_0 plant and determined the presence or absence of the transgene in 42 plants from the T_1 progeny by Southern blots. The plants carrying the transgene flowered on average 42 days earlier than the negative plants ($P<0.001$, Fig. 3A-B). Quantitative PCR analysis of eight negative and eight positive transgenic T_1 plants (Fig. 3C-F) showed reduction of the endogenous RNA levels of *ZCCT1* ($P<0.05$, Fig. 3D) but not of *ZCCT2* ($P=0.79$, Fig. 3E). The positive transgenic T_1 plants showed higher *AP1* RNA levels ($P<0.001$, Fig. 3F). This experiment confirmed that the reduction of the RNA level of *ZCCT1* is associated with the acceleration of flowering time and that RNAi can be used successfully in polyploidy wheat, which carries multiple homoeologous copies of *ZCCT1*. This experiment demonstrated that *VRN2* also regulates flowering by vernalization in polyploid winter wheats, despite observations of allelic variation at this locus in diploid wheat but not in polyploid wheat (4).

The vernalization response in temperate cereals

The complete linkage between *ZCCT1* and *VRN2* in a large mapping population, its gradual and stable transcriptional downregulation by vernalization, its opposite transcription profile to *VRN1*, the association between natural allelic variation at *ZCCT1* and spring growth habit, and the acceleration of flowering time by RNAi of *ZCCT1* transcripts all demonstrate that *ZCCT1* is *VRN2*.

The *ZCCT1* gene belongs to a different family of transcription factors than the *Arabidopsis* MADS-box gene *FLC* but has an analogous function. Both genes are dominant repressors of flowering downregulated by vernalization. Similarities between *ZCCT1* and Arabidopsis proteins are restricted to the conserved CCT domains present in *CO* and *CO-like* proteins.

However, the CCT domains from the *ZCCT* genes belong to a group that does not include any Arabidopsis protein (SOM-3, Fig.S11).

An additional difference between wheat and Arabidopsis is the frequent association between natural differences in growth habit and allelic variation at the *AP1* locus in wheat (5), but not in Arabidopsis. Even in the extensive collection of Arabidopsis mutants there are no reports of differences in vernalization requirement associated with the *AP1* gene.

Based on the previous observations, and the knowledge that temperate grasses evolved from subtropical primitive grasses that likely had no vernalization requirement (20), we conclude that Arabidopsis and the temperate grasses developed different vernalization pathways, including different genes downregulated by vernalization (*ZCCT1* and *FLC*) and similar genes with different regulatory profiles (*AP1*).

The development of a vernalization response was an important step for the adaptation of the grasses to the cold regions. In most of the wild Triticeae species vernalization accelerates flowering, suggesting that the winter growth habit is the ancestral state in this group of species. However, these winter species retained the capacity to generate spring forms by loss of function mutations at two main vernalization loci. Independent mutations at these loci were maintained by natural selection in the wild species and by a strong selection pressure in the domesticated wheat and barley varieties. These results suggest that the wide adaptability of temperate cereals was favored by a flexible regulation system of flowering time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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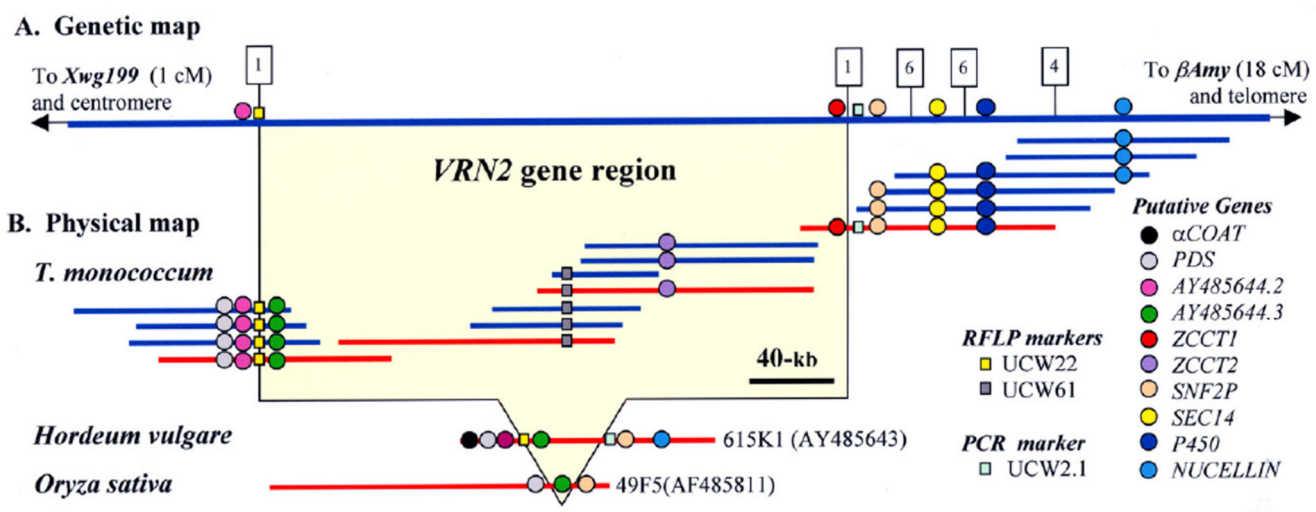

Fig. 1.

A. Genetic map of the *VRN2* region on chromosome 5A^{m} of *T. monococcum* based on 5,698 gametes. Numbers of crossovers in the critical recombinant plants are indicated in boxes. **B.** Physical map of the wheat *VRN2* region in *T. monococcum* and in colinear regions from barley and rice. BAC clones indicated in red have been sequenced (438,828-bp, AY485644). The order of BAC clones from left to right is: 374A18, 94E8, 304H18, **258C22, 301G15**, 615O6, 650N20, **405L8**, 271O11, 275P20, 157P20, **455C17**, 322L23, 702K8, 32A1, 533H16 and 324G2 (bold letters indicate sequenced BACs). Additional information for the markers used in this figure has been deposited in the SOM.

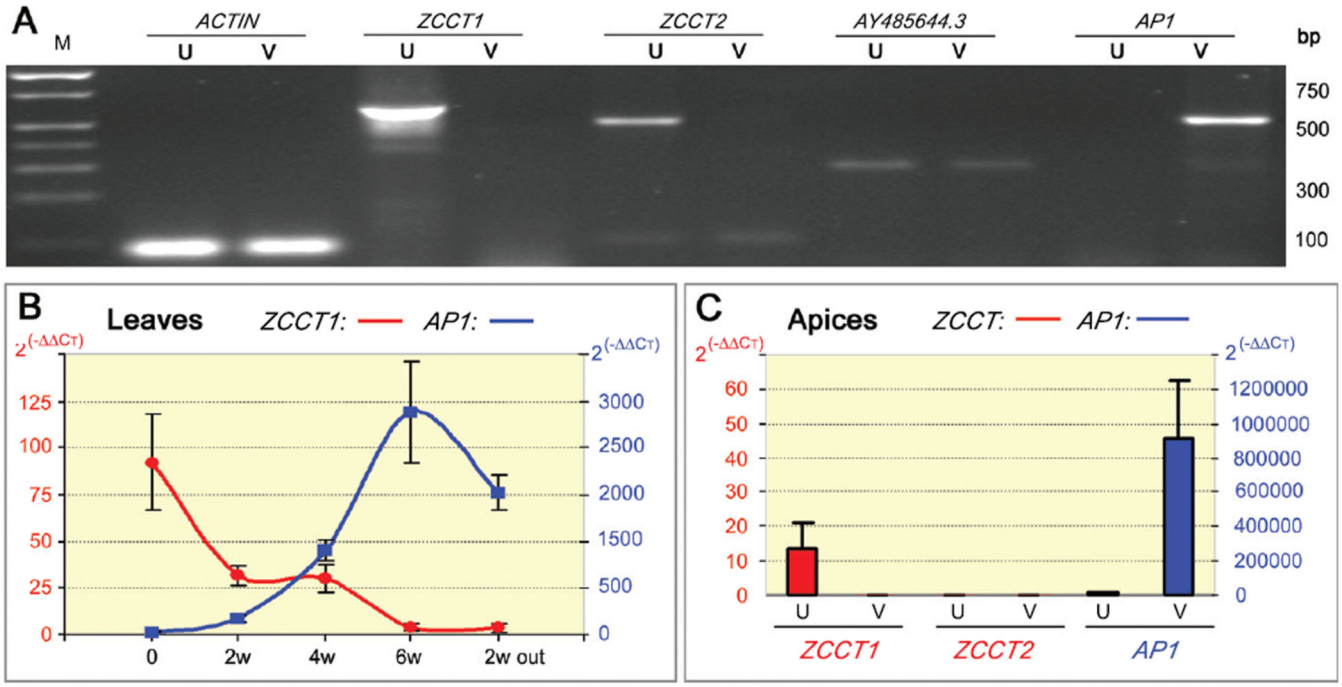

Fig. 2.

A. RT-PCR from leaves of unvernalized (U) or vernalized (V) G3116 winter wheat plants. RNA samples from the vernalized plants (6 weeks at 4°C) were collected 5 days after returning the plants to room temperature. Among the three genes completely linked to *VRN2*, RNA levels of *ZCCT1* and *ZCCT2* were down regulated by vernalization and those of AY485644.3 were not affected by vernalization. In the same cDNA samples, *AP1* RNA levels were up regulated by vernalization. M indicates molecular weight DNA marker. B-C) Quantitative PCR. **B.** ***Leaves***: Transcript levels of *ZCCT1* (red scale) and *AP1* (blue scale) relative to *UBIQUITIN* in G3116 (averages of 5 plants ± SE): 0: before 4°C; 2w, 4w, 6w: weeks at 4°C; 2w out: 2 weeks at room temperature after vernalization. **C.** ***Apices***: Transcript levels of *ZCCT1*, *ZCCT2* and *AP1* (=*VRN1*) relative to *ACTIN* in G3116 (averages of 3 pools of apices from 5 plants each ± SE). U= unvernalized, V= 3–5 days at room temperature after 6 weeks of vernalization. Units are linearized values using the $2^{(-\Delta\Delta C_T)}$ method, where CT is the threshold cycle.

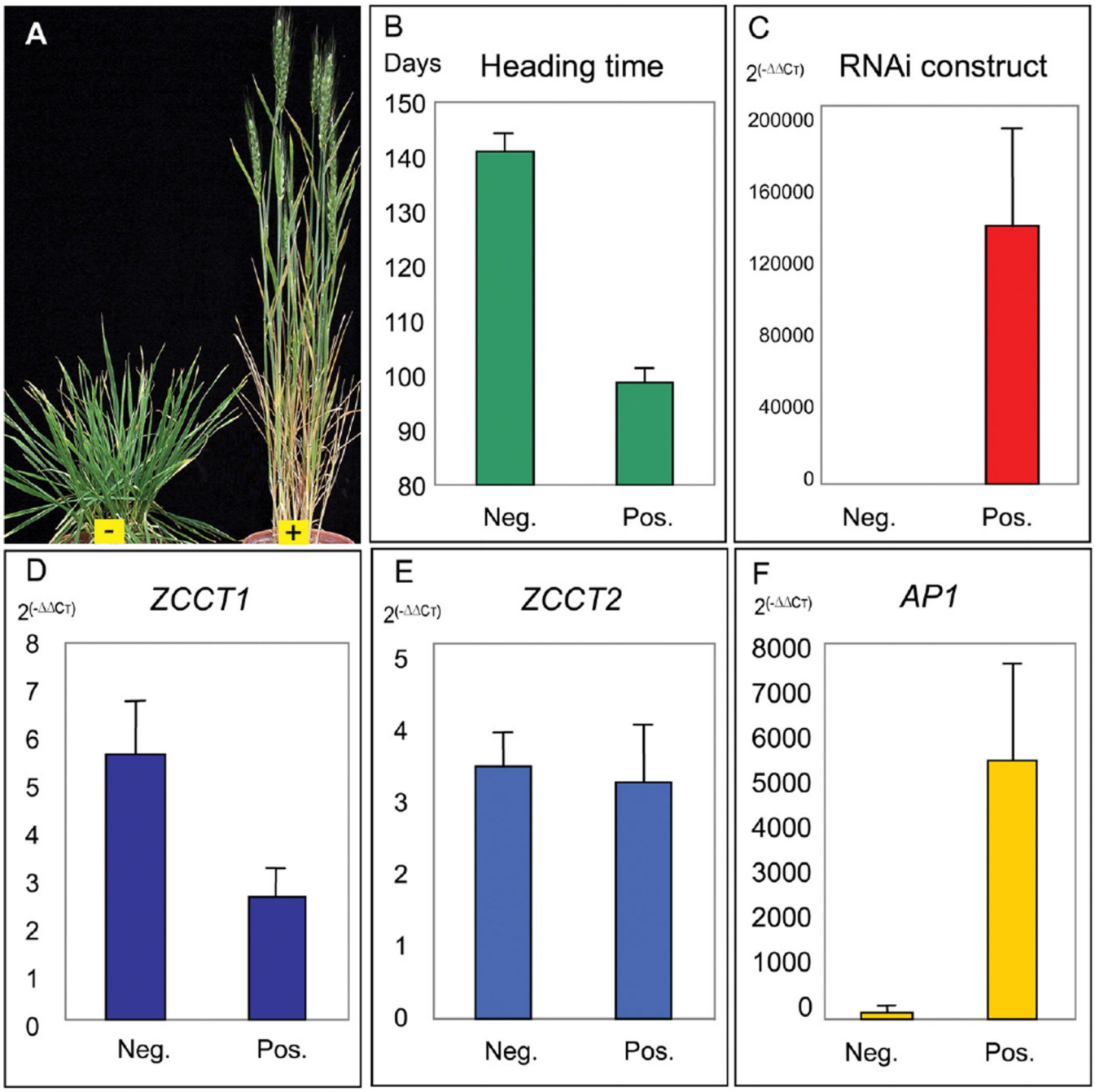

Fig. 3.

A. Transgenic Jagger T_1 plants segregating for the presence and absence of the RNA interference construct for *ZCCT1* and for flowering time. **B.** Average heading date of T_1 plants carrying the transgene (31 plants) and without the transgene (11 plants). **C-F.** Average RNA level of 8 positive and 8 negative T_1 plants from the progeny of the early flowering T_0 plant. Units are linearized values using the $2^{(-\Delta\Delta C_T)}$ method, where CT is the threshold cycle. **C.** RNA level of the RNAi construct; **D-F.** Endogenous RNA levels of **D.** *ZCCT1*, **E.** *ZCCT2*, **F.** *AP1*. Error bars indicate one standard error of the mean.